CANCER CHEMOPREVENTION BY FOOD PHYTOCHEMICALS REGULATING INFLAMMATORY LEUKOCYTES(Dissertation_全文)

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CONTENTS

ABBREVIATIONS

ACA	1'-Acetoxychavicol acetate
AUR	Auraptene
CUR	Curcumin
DCF	2',7'-Dichlorofluorescein
DCFH-DA	2',7'-Dichlorofluorescin diacetate
DHTHC	Dihydroxytetrahydrocurcumin
DMBA	7,12-Dimethylbenz[*a*]anthracene
EBV-EA	Epstein-Barr virus-early antigen
EIMS	Electron impact mass spectrometry
GEN	Genistein
GPx	Glutathione peroxidase
H_2O_2	Hydrogen peroxide
HC	1'-Hydroxychavicol
HCA	1'-Hydroxychavicol acetate
HPLC	High performance liquid chromatography
HRPO	Horseradish peroxidase
LAH	Lithium aluminum hydride
LIS	Lanthanide-induced shift
5-LO	5-Lipoxygenase
MeACA	1'-Acetoxy-1'-methylchavicol acetate
MPO	Myeloperoxidase
MTPA	α-Methoxy-α-trifluoromethylphenylacetate
NBT	Nitroblue tetrazolium
4-NQO	4-Nitroquinoline 1-oxide
O_2^-	Superoxide
PKC	Protein kinase C
PLA_2	Phospholipase A_2
PMN	Polymorphonuclear leukocytes
PPBa	Pheophorbide *a*
ROS	Reactive oxygen species
SBL	Soybean lipoxygenase
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reacting substances
THC	Tetrahydrocurcumin
TLC	Thin layer chromatography
TPA	12-*O*-Tetradecanoylphorbol-13-acetate
UMB	Umbelliferone
XA/XOD	Xanthine/xanthine oxidase

Chapter I:
General Introduction

Evidence for Lowered Cancer Risks by Ingestion of Food Phytochemicals

A body of epidemiological surveys and animal experiments have demonstrated that ingestion of some constituents occurring in vegetables and fruit may contribute to the reduction of cancer incidence in humans (Bertram 1987, Wattenberg 1985). In 1991 and 1992, comprehensive reviews on the possible protective effect of the consumption of vegetables and fruit were published (Steinmetz 1991a,b, Block 1992). It was concluded that consumption of higher levels of vegetables and fruit is associated consistently, although not universally, with a reduced cancer risk at most sites in the body, and that the association is most marked for epithelial cancers. Epidemiological evidence about the effect of particular kinds of vegetables or fruit and their constituents has been demonstrated to draw consistent inferences, for examples of inverse associations between raw tomato intake and the risk of digestive cancer, which point to a protective role of lycopene, a major carotenoid in tomatoes, and ascorbic acid (Franceschi 1994). On the other hand, at least 20 studies in which cancer has been experimentally induced via a chemical carcinogen or irradiation in mice, rats, or hamsters and the animals were fed specified amounts of certain vegetables and fruits have been conducted (Diet & Cancer Project 1997). In the great majority of these studies, it was found that the animals fed vegetables or fruit experienced fewer tumors, smaller tumors, less DNA damage, higher levels of detoxification enzymes, or other outcomes indicative of a lower risk of cancer. In most studies, the relative amounts of vegetables included in animal diets were well above those typically consumed by humans. Vegetables and fruits all contain a variety of constituents thought to protect against cancer. Especially, microconstituents, likely to play important roles in protecting against cancers on the basis of these results of animal studies, include the antioxidative vitamins, carotenoids and flavonoids. Thus, at present, when a reliable method for cancer therapy has not yet been established, cancer chemoprevention by these food phytochemicals is accepted as an attractive and promising avenue for cancer control (Lippman 1994).

Reactive Oxygen Species in Mouse Skin

Reactive oxygen species (ROS) induce membrane damage, DNA base oxidation, DNA strand breaks, chromosomal aberrations, and protein alterations, most of which would be involved in carcinogenesis processes (Cerutti 1985). At present, a mounting evidence indicating the importance of oxidative stress has been widely accepted in the mechanisms of tumor promotion. This mechanism is recognized as a long-term and reversible step that converts dormant tumor cells (already initiated cells) into visible tumor cells; the classical hypothesis of chemical carcinogenesis, or so-called "two-stage (initiation/promotion) carcinogenesis theory" (Berenblum 1941). For instance, organic peroxides and free-radical generators have tumor-promoting activities in the mouse, and inversely antioxidants including radical scavengers inhibit several biological and biochemical effects of tumor promoters and also tumorigenesis in rodents (Perchellet 1995). Skin tumor promoters such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) are known to show a variety of biological activities in mouse skin or other biological systems (Lin 1995). Among these activities, it is noteworthy that TPA enhances the generation of ROS and causes a decline in ROS-detoxificating enzymes in both epidermal and inflammatory cells. In a mouse skin model, TPA triggers ROS accumulation through the activation of the xanthine oxidase (XOD) system or stimulation of polymorphonuclear leukocytes (PMN) (Perchellet 1995) and the inhibition of catalase or glutathione peroxidase (GPx) activity (Solanki 1981). ROS are also generated enzymatically during the TPA-induced arachidonate metabolism.

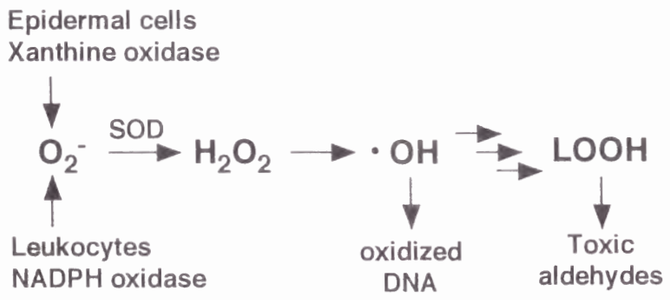

Fig. I-1 Reactive oxygen species in mouse skin

Yoon *et al.* (1993) advocated a close relationship between the generation of ROS by phagocytic cells in inflammatory processes and tumor promotion. Kensler *et al.* (1987, 1989) have proposed the hypothesis that treatment of mouse skin with the first TPA application causes a chemotactic action, i.e., recruitment of neutrophils responsible for ROS generation induced by a second TPA treatment. In fact, double applications of TPA are required for excessive ROS production in mouse skin (Wei 1992). Ji *et al.* (1992) concluded that each application triggers two distinguishable biochemical events, termed as priming and activation (Fig. I-2). The former event has been recognized mainly as recruitment of neutrophils, while the latter as the stage of ROS production in neutrophils, keratinocytes, etc. Moreover, ROS production by double or multiple TPA treatments is closely associated with the metabolic activation of proximate carcinogens (Ji 1992, Kensler 1987, 1989) and the increased levels of oxidized DNA bases (Wei, 1991, 1992). The evidence for participation of inflammatory oxidative damage has been accepted in epithelial cancer including not only skin cancer but also oral (Thomas 1995), lung (Petruska 1992) and colon cancer (Grisham 1992). On the above view points, suppression of ROS generation in inflammatory processes is regarded as one of the most effective strategies for cancer chemoprevention.

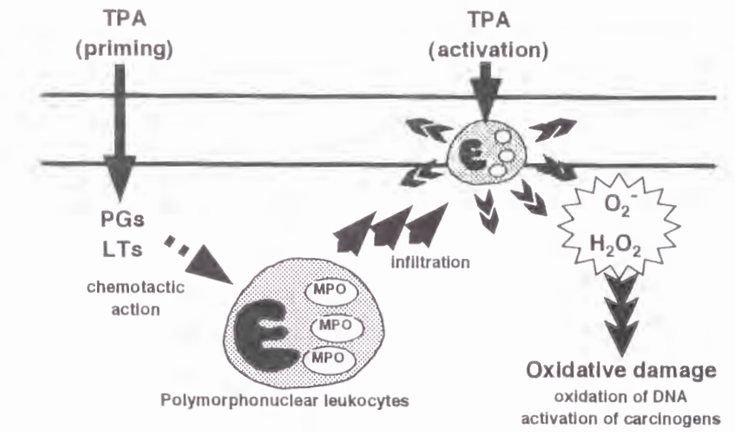

Fig. I-2 Proposed mechanism of ROS generation by double TPA application in mouse skin

Antioxidants in Cancer Chemoprevention

Antioxidants in biological systems can be classified, at least, into three groups on the basis of their action mechanisms, e.g., 1) inhibitors of ROS generating enzyme induction, for instance, the expression of proteins, assembly of the enzyme components, etc., 2) inhibitors of enzyme activities, and 3) radical scavengers reacting directly with ROS. Of these groups, radical scavengers have mainly been noticed as the most promising candidate for chemopreventers because they have been found to strongly inhibit oxidative reactions *in vitro* and *in vivo*. Therefore dietary radical scavengers, such as α-tocopherol, ascorbic acid, β-carotene and simple phenolics have hitherto attracted wide attention in recent years (Huang 1992a). These compounds indeed have antimutagenic and anti-tumor promoting activities and possess several other biological properties, possibly related to anti-carcinogenesis.

Among these dietary phytochemicals, β-carotene, a major carotenoid occurring widely in green-yellowish vegetables and fruits, is one of the most extensively studied agents for chemoprevention on account of its low toxicity and cancer preventive potencies in various animal models and the epidemiological surveys (Malone 1991, Peto 1981). Several human intervention trials using β-carotene were conducted or are ongoing in the USA, e.g., The Physicians' Health Study, Women's Health Study (Buring 1995), or CARET study (Omenn 1994) (Table I-1). A puzzling outcome, however, was reported that β-carotene failed in reducing cancer risks and mortalities in a recent clinical study (The ATBC Group 1994). To date, no beneficial effects of β-carotene in terms of chemoprevention have been reported except for the case of the Linxian Study (Blot 1993). It is evident that β-carotene is a mere phytochemical not exclusively representing the cancer preventive potential of vegetables. Moreover, some radical scavengers exert not only weak anti-tumor promoting activity but also carcinogenic activity in rodents when given at high doses (Ito 1989, Weisburger 1992). Radical scavengers are known to have prooxidative potential because of their conversion to more reactive or stable radicals after they react directly with ROS, which may contribute to the induction of secondary oxidative damage on the target organs. Hence, there may be a need to discover new types of chemopreventive agents which have antioxidative properties rather than those acting as radical scavengers, i.e.,

generation inhibitors, by scrutinizing a diverse variety of edible plants and their components.

Table I-1 Summary of human intervention trials using β-carotene in the USA

Trial	Population/ country	Agents	Outcome
Linxian Study (1986-1991)	29,584 China	β-carotene+ α-tocopherol+ selenium	lung cancer 21% ↓
ATBC Study (1985-1993)	29,133 Finland	β-carotene+ α-tocopherol	lung cancer 18% ↑
Physicians' Health Study (1982-1995)	22,071 USA	β-carotene+ aspirin	no beneficial or harmful effects
CARET Study (1988)	18,314 USA	β-carotene+ all-trans-retinol	lung cancer 28% ↑
Women's Health Study (1992~)	Ca. 40,000 USA	β-carotene+ (ongoing) α-tocopherol+ aspirin	-

Study Concept and Outlines

In this context, the author proposes a hypothesis that ROS generating inhibitors, including both enzyme induction and enzyme activity inhibitors, must suppress oxidative stress and hence tumorigenesis in rodents more effectively than radical scavenging-type antioxidants, because the former can inhibit generation of some types of ROS at earlier stages and may not allow the following oxidative damage to cascade. In this thesis, the author demonstrates that regulation of leukocytes in inflammatory regions is one of the most effective strategies for oxidative stress control. In Chapter II and III, inhibitory effects of 1'-acetoxychavicol acetate (ACA), a potent chemopreventer of several organ carcinogeneses, on ROS generation in leukocytes *in vitro* and *in vivo* are examined. Further its strong inhibition of oxidative damage in mouse skin and consistent evidence for suppression of

leukocyte activation in inflammatory regions by ACA are demonstrated. In Chapter IV, inhibitory effects of some food phytochemicals on ROS generation by leukocytes in mouse skin are examined and can be categorized into three classes by their inhibitory profiles in the priming and/or activation phase in inflammation processes caused by TPA. Finally isolation and identification of new ROS generation inhibitors from *Artemisia lactiflora* are highlighted (Chapter V), their inhibitory effects on TPA-induced oxidative stress and tumor promotion in mouse skin are also described in Chapter VI.

Chapter II:
Inhibitory Effects of 1'-Acetoxychavicol Acetate from Greater Galangal on Reactive Oxygen Species Formation

Introduction

In previous studies on the search for new types of chemopreventive agents from edible plants in southeast Asia (Kondo 1993, Murakami 1993, 1994, 1995a,b, 1996a, 1998a,b,c, Nakamura 1996a,b), 1'-acetoxychavicol acetate (ACA, Fig. II-1), isolated from the rhizomes of a Zingiberaceae plant, greater galangal (*Languas galanga*), was found to be a potent inhibitor of tumor promoter-induced Epstein-Barr virus (EBV) activation (Kondo 1993). ACA was later reported to be a potent inhibitor of chemically induced carcinogeneses in several rodent models (Table II-1).

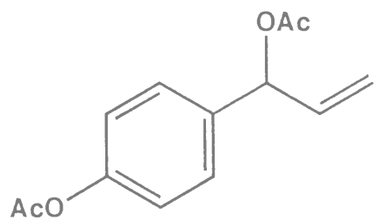

Fig. II-1 Chemical structure of ACA

Table II-1 Chemopreventive effects of ACA on chemically induced carcinogeneses in rodent models

Organ	Carcinogen	Administration	Ref.
Skin	DMBA[b]	Topical	Murakami 1996b
Tongue	4-NQO[c]	Oral	Ohnishi 1996
Colon (ACF[a])	AOM[d]	Oral	Tanaka 1997a
Colon (Cancer)	AOM[d]	Oral	Tanaka 1997b
Liver	CDAA diet[e]	Oral	in preparation

[a]ACF: aberrant crypt foci
[b]DMBA: 7,12-dimethylbenz[a]anthracene
[c]4-NQO: 4-nitroquinoline 1-oxide
[d]AOM: azoxymethane
[e]CDAA diet: a choline-deficient, L-amino acid-defined diet

For example, in a two stage mouse skin carcinogenesis experiment, topical application of ACA even at an equimolar dose to TPA during the promotion stage significantly reduced tumor formation in 7,12-dimethylbenz[*a*]-anthracene (DMBA)-initiated mouse skin (Murakami 1996b). The anti-tumor promoting activity of ACA in mouse skin was much higher than those of well-known chemopreventers from edible plants such as quercetin (Nishino 1984a), genistein (Wei 1995), glycyrrhetic acid (Nishino 1984b), and (-)-epigallocatechin gallate (Yoshizawa 1987). In the following study of 4-nitroquinoline 1-oxide (4-NQO)-induced rat tongue carcinogenesis, rats fed on a diet containing ACA at 100 ppm in the initiation or post-initiation phase bore no tumors whereas 58% of the rats in the control group did bear tumors (Ohnishi 1996). Chemopreventive activity of ACA in this model is also prominent as compared with those of difluoromethylornithine (Tanaka 1993), β-carotene (Tanaka 1994) and curcumin (Tanaka 1994). ACA has lately been found to suppress azoxymethane-induced rat colonic aberrant crypt foci (ACF) formation (Tanaka 1997a) and tumorigenesis (Tanaka 1997b). In addition, ACA suppressed the formation of a tumor marker γ-glutamyltransferase in the liver of rat fed with a choline-deficient, L-amino acid-defined (CDAA) diet (Kobayashi *et al.* in preparation).

Table II-2 Summary of mechanistic studies on ACA

Inhibition of:	
EBV activation	+++
O_2^- generation in differentiated HL-60 cells	+++
XOD	+
autoxidation of ethyl linoleate	±
PDBu[a] binding to the membrane fraction	–
PKC activation	–
5-,12-lipoxygenase	–
cyclooxygenase	–
inflammation in mouse skin	–
arachidonic acid release from cell membrane	–
Pi incorporation to cell membrane	–

[a]PDBu: phorbol-12,13-dibutyrate

The exact mechanism by which ACA exhibits its chemopreventive action in rodent models, however, remains to be clarified in detail (Table II-2). ACA was previously reported as an inhibitor of XOD (Noro 1988), which catalyses hydroxylation of many purine substances and generates superoxide (O_2^-). This was reconfirmed by the author *et al.* (Murakami 1996b). It should be noted that ACA potently inhibits TPA-induced O_2^- generation in differentiated HL-60 cells (Murakami 1996b), while ACA is a much weaker antioxidant than α-tocopherol in the linoleate autoxidation system and has no O_2^- scavenging potential (Murakami 1996b). Taken together, ACA is recognized to be a O_2^- generation inhibitor but never such a radical scavenger as phenolic antioxidants, lacking phenolic hydroxyl groups, supported by its chemical structure.

In this chapter, synthesis of ACA derivatives are described, and their anti-tumor promoting potentials and inhibitory effects on ROS formation *in vitro* are characterized. The structure-activity relationship of ACA analogs in the bioassays relating to ROS generation are then examined to find which stage of the ROS generation cascade (Fig. I-1) is important for the anti-tumor promoting activity of ACA.

Results

Preparation of ACA Analogs and Their In Vitro *Anti-tumor Promoting Activities*

As outlined in Fig. II-2, ACA, 1'-hydroxychavicol acetate (HCA) and 1'-hydroxychavicol (HC) were synthesized as reported previously (Kondo 1993). 1'-Acetoxy-1'-methyl-chavicol acetate (MeACA) was synthesized from *p*-hyroxyacetophenone.

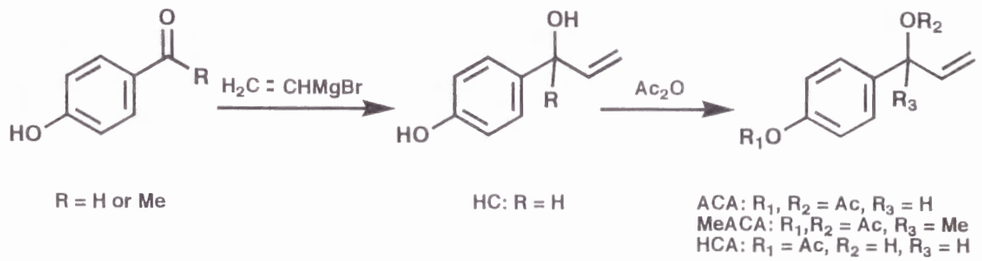

Fig. II-2 Synthesis of ACA analogs

ACA analogs were tested for inhibitory activities of tumor promoter-induced Epstein-Barr virus (EBV) activation in Raji cells and O_2^- generation in differentiated HL-60 cells. The values of the half-maximal inhibitory concentration (IC_{50}) are shown in Table II-3. ACA strongly inhibited EBV activation and O_2^- generation as previously reported (IC_{50} = 1.3 μM and 4.3 μM, respectively). MeACA showed moderate inhibitory activities (IC_{50} = 26 μM and 29 μM, respectively), while HCA and HC even at a concentration of 100 μM were inactive in the EBV and O_2^- generation assays.

In Vitro *Antioxidative Activities of ACA Analogs in Cell-free Systems*

Antioxidative effects of ACA derivatives were examined by the lipid peroxidation and XA/XOD systems. As shown in Table II-4, HC bearing two hydroxyl groups showed the strongest antioxidative activity among the test compounds in the lipid peroxidation assay (IE = 66 %, $P > 0.005$ *versus* ACA), while other compounds showed weak antioxidative activities (ACA; IE = 33%, MeACA; IE = 30%, HCA; IE = 27%). On the other hand, HCA exhibited no inhibitory activity in the XA/XOD system up to 100 μM whereas ACA, MeACA and HC showed moderate inhibitions (IE = 61%, 57%, and 44%, respectively). All compounds tested did not scavenge O_2^- up to 100 μM in the XA/XOD system (data not shown).

Table II-3 Inhibitory activities of ACA analogs against tumor promoter-induced EBV activation in Raji cells and O_2^- generation in differentiated HL-60 cells[a]

	IC_{50} (μM)	
Compound	EBV	O_2^-
ACA	1.3	4.3
MeACA	26	29
HCA	>100	>100
HC	>100	>100

[a]Mean value of the half maximal inhibitory concentration obtained from at least duplicate experiments. The maximal standard deviation (SD) for each experiments was 5%.

Table II-4 *In vitro* antioxidative activities of ACA analogs in the XA/XOD system and linoleate autoxidation system[a]

Compound	XOD[b]	TBARS[c]
ACA	61 ± 2	33 ± 2
MeACA	57 ± 5	30 ± 5
HCA	4 ± 3[d]	27 ± 5
HC	44 ± 2[d]	66 ± 2[d]

[a]Data are expressed from at least two experiments.
[b]Mean ± SD (n = 2).
[c]TBARS: thiobarbituric acid reacting substances. Mean ± SD (n = 2).
[d]Significantly different from ACA ($P < 0.001$, Student's *t*-test).

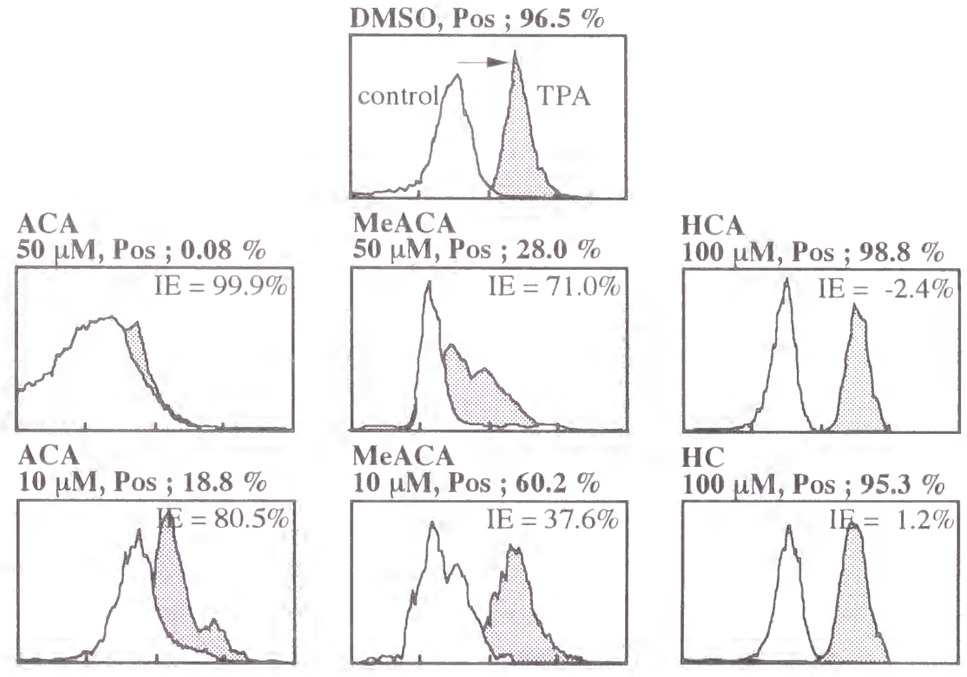

Fig. II-3. DCF fluorescence distribution in DMSO-differentiated HL-60 cells. Cells were preincubated with 50 μM DCFH-DA at 37°C for 15 min. After being treated with DMSO (positive control) at 37°C for 15 min, the cells were treated with EtOH (unstimulated control, left), or 100 nM TPA (right). As for inhibition of ACA analogs, cells were preincubated with 50 μM DCFH-DA at 37°C for 15 min. After being treated with the test compound at 37°C for 15 min, the cells were treated with EtOH (left), or 100 nM TPA (right). The DCF fluorescence was monitored on a flowcytometer (CytoACE 150) with excitation and emission wavelengths at 488 nm and 600 nm, respectively.

Inhibitory Effect of ACA Analogs on Intracellular Peroxide Formation in Differentiated HL-60 Cells

Subsequently, inhibitory activities against the formation of peroxides including hydrogen peroxide (H_2O_2) and hydroperoxides in differentiated HL-60 cells were determined by using 2',7'-dichlorofluorescin diacetate (DCFH-DA) as an intracellular fluorescence probe. As shown in Fig. II-3, most of the cells were estimated to produce peroxides ("Peroxide Positive Rate (PPR)" = 97%) with TPA stimulation alone. ACA at a concentration of 10 μM significantly inhibited peroxide formation by 81%. PPR and inhibitory effects of test compounds are showed in Fig. II-3. MeACA at 10 μM exhibited weaker suppressive effect (IE = 38 %) than ACA, and both HC and HCA were inactive up to a concentration of 100 μM.

Discussion

Potent chemopreventive activities of ACA in mouse skin, rat tongue and colon have previously been reported (Murakami 1996b, Ohnishi 1996, Tanaka 1997a,b). The action mechanism(s) by which ACA inhibits TPA-induced tumor promotion in mouse skin is unclear since ACA affects neither the specific binding of [^{3}H]phorbol-12,13-dibutyrate to the particulate fraction of mouse skin nor protein kinase C (PKC) activity (Watanabe 1995). However, ACA has recently been found to be a considerable O_2^- generation inhibitor in both the XA/XOD system and differentiated HL-60 cells (Murakami 1996b). These findings have led the author to assume that ACA inhibits carcinogenesis by reduction of oxidative stress possibly through suppression of ROS generation pathways in biological systems. In order to clarify the action mechanism(s) of ACA for its anti-tumor promoting effects, the author studied the structure-activity relationships of ACA and its analogs since the structure of ACA is simple enough to synthesize and modify its functional groups. Inactive analogs of ACA in each *in vitro* activity were thought to be useful for clarification of which biological activity essentially contributes to the anti-promotional effects *in vivo*. The author therefore conducted some *in vitro* assays for inferring of anti-tumor promoting potentials of ACA and its analogs. The author first selected the EBV activation assay because EBV, known to be causative of African Burkitt's lymphoma (Klein 1985) and anaplastic nasopharyngeal

carcinoma (Young 1988), and presumably for gastric cancer in part (Lorenzo 1993), is demonstrated to be activated by tumor promoters and the inhibitors of this activation have been proven to be chemopreventers of TPA-induced tumor promotion in mouse skin as well as in other organ carcinogenesis models (Murakami 1995b, 1996b, 1997, Nakamura 1996b, Ohigashi 1997, Ohnishi 1996, Tanaka 1997a,b,c, 1998). The author also conducted the inhibitory assay of TPA-induced O_2^- generation in human promyelocytic leukemia HL-60 cells as a model of the NADPH oxidase system. By treatment of HL-60 cells with dimethylsulfoxide (DMSO) for 5 days, they are differentiated into granulocytes with an increment in expression of the components of the NADPH oxidase responsible for TPA-induced O_2^- generation (Henderson 1996). The author and other groups have recently reported that some natural chemopreventers inhibit O_2^- generation, which are suggested, at least in part, to be important action mechanisms (Murakami 1996b, 1997, Nakamura 1996b, Wei 1992, 1993a). Furthermore, *in vitro* antioxidative assays using both the XA/XOD and lipid peroxidation systems were conducted since some radical scavengers are well-known to inhibit biochemical and biological effects of tumor promoters, linking anti-tumor promotion (Perchellet 1995).

As shown in Tables II-3 and 4, HC, having two hydroxyl groups which generally play essential roles for radical scavenging or metal ion chelating effect of phenolic antioxidants, showed the highest inhibitory activities against thiobarbituric acid reacting substances (TBARS) formation, an overall marker of lipid peroxidation, as compared with those of other compounds. HC has no inhibitory effect on tumor promoter-induced EBV activation and O_2^- generation (Table II-3) while it showed a strong antioxidative effect on the propagation of lipid peroxidation (Table II-4). These activity profiles are inverse to those of ACA, i.e., potent inhibition of EBV activation and O_2^- generation and week anti-peroxidative activity (Tables II-3 and 4). In the XA/XOD system, HC exhibited significant XOD inhibitory activity (Table II-4) and no O_2^- scavenging activity (data not shown). This is similar to ACA. Thus, HC was selected as the most appropriate tool among the ACA analogs for *in vivo* antioxidative studies.

To estimate the inhibitory potential of ACA on TPA-induced oxidative events *in vivo*, the author has conducted inhibitory assays of intracellular peroxide formation using DCFH-DA as an intracellular

fluorescence probe. Suppression of peroxide formation in differentiated HL-60 cells by ACA (Fig. II-3) is expected to be attributable to the inhibition of O_2^- generation since a certain portion of intracellular peroxides is considered to be originally and indirectly formed by O_2^- in differentiated HL-60 cells. Inhibitory effects of ACA and its analogs on O_2^- generation were not due to their O_2^- scavenging effect since these compounds were washed out before TPA stimulation in the experimental condition (see **Experimental**). Also, it is noticeable that ACA exhibited neither O_2^- nor H_2O_2 scavenging effects (data not shown).

Table II-5 Inhibitory activities of ACA analogs against tumor promoter-induced EBV activation and ROS generation in differentiated HL-60 cells

	IC_{50} (μM)		
Compound	EBV	O_2^-	Peroxide
ACA	1.3	4.3	2.9
MeACA	26	29	16
HCA	>100	>100	>100
HC	>100	>100	>100

Furthermore, good correlation of effective concentrations of ACA derivatives between inhibition of O_2^- generation and reduction of peroxide levels (Table II-5) may support this assumption. Takeuchi *et al.* (1996) have indicated that extracellular O_2^- of HL-60 cells may be generated by plasma membrane-bound NADPH oxidase and diffuse into the cells through the anion channel but intracellular H_2O_2 may be formed extracellularly from O_2^-, being diffused into the cells through membrane. Lundqvist *et al.* (1996) also confirmed the origin of intracellular H_2O_2 by the observation that differentiated HL-60 cells, lacking of specific granules in which all membrane components necessary for NADPH oxidase activity are present, showed much lower intracellular chemiluminescence response than the isolated neutrophils having specific granules. These findings also support the idea that ACA suppresses intracellular peroxide formation due to inhibition of plasma membrane-bound NADPH oxidase. The exact mechanism for the inhibition of NADPH oxidase activity by ACA remains to be clarified. It should be noted that staurosporine, a microbial alkaloid PKC inhibitor without antioxidantive activity, inhibits O_2^- production in

neutrophils (Yamamoto 1989). As described above, it is interesting that ACA does not alter PKC activity, suggestive of the existence of novel pathway(s) for inhibition of the NADPH oxidase system. In any case, more extensive mechanistic studies on the inhibition of TPA-induced O_2^- generation by ACA are necessary.

In summary, the good structure-activity relationships of ACA analogs between EBV activation inhibition, O_2^- generation and intracellular peroxide formation in HL-60 cells were observed. These results suggested that inhibition of O_2^- generation in leukocytes, at least in part, might be an important action mechanism for the suppression of oxidative stress and tumor promotion by ACA in mouse skin.

Experimental

Chemicals

Teleocidin B-4 was isolated from *Streptoverticillium blastmyceticum* NA 34-17 as previously reported (Irie 1984). TPA was obtained from Research Biochemicals International, MA, USA. RPMI 1640 medium and fetal bovine serum were purchased from Gibco RBL, NY, USA. DCFH-DA was obtained from Molecular Probes, Inc., Leiden, The Netherlands. Cytochrome *c* and ethyl linoleate were obtained from Sigma, MO, USA. High-titer EBV early antigen (EBV-EA)-positive sera from anaplastic nasopharyngeal carcinoma patients were kindly gifted from Prof. Dr. Ohsato (Health Sciences University of Hokkaido). FITC-labeled anti-human IgG was obtained from Dako, Glostrup, Denmark. All other chemicals were purchased from Wako Pure Chemical Industries, Osaka, Japan.

Synthesis of ACA and Its Derivatives

ACA, HCA and HC were synthesized as previously reported (Kondo 1993). MeACA was synthesized from *p*-hyroxyacetophenone as outlined in Fig. II-2. A solution of *p*-hydroxyacetophenone (108 mg) in 40 ml of dry tetrahydrofuran was added to 1.0 M vinylmagnesium bromide in 100 ml of dry tetrahydrofuran under N_2 atmosphere for 2 h. After removal of THF *in vacuo*, the reaction mixture was partitioned between ethyl acetate and NH_4Cl-saturated water to give 1'-hydroxy-1'-methyl-chavicol, which was

then acetylated with 4-dimethylaminopyridine and acetic anhydride. The reaction mixture was purified by preparative TLC (toluene: acetone, 9:1) and preparative HPLC (column: μBondasphere C_{18} 19 mm x15 cm, mobile phase: 40% acetonitrile in H_2O, flow rate: 7.0 ml/min, detection: 254 nm) to give MeACA (24 mg). UV λmax (EtOH) nm (ε): 255 (152); IR νmax (polyethylene film) cm^{-1}: 1770, 1740, 1510, 1370, 1220, 1200, 1170, 1020. ^{1}H-NMR (300 MHz, $CDCl_3$). δ: 1.87 (3H, *s*, 1'-Me), 2.06 (3H, *s*, 1'-OAc), 2.28 (3H, *s*, 4-OAc), 5.24 (1H, *dd*, *J* = 10.8, 0.5 Hz, H-3'a), 5.26 (1H, *dd*, *J* = 17.4, 0.5 Hz, H-3'b), 6.23 (1H, *dd*, *J* = 17.4, 10.8 Hz, H-2'), 7.05 (2H, *d*, *J* = 8.7 Hz, H-3,5), 7.38 (2H, *d*, *J* = 8.7 Hz, H-2,6). EIMS (probe, 70eV) *m/z*: 248 ($[M]^+$, $C_{14}H_{16}O_4$).

Inhibitory Test of EBV Activation

Human B-lymphoblastoid Raji cells were incubated in 1 ml of RPMI 1640 medium (supplemented with 10% fetal bovine serum) containing sodium *n*-butyrate (3 mM), teleocidin B-4 (50 nM) and a test compound in DMSO at 37°C under 5 % CO_2 atmosphere for 48 h. EBV activation was estimated by detection of EA using an indirect immunofluorescence method (Murakami 1997). The smears were made from cell suspension and stained with high-titer EA-positive sera followed by fluorescein isothiocyanate (FITC)-labeled IgG. The rate of EA-inducing cells was compared with that of a control experiment only with sodium *n*-butyrate, teleocidin B-4 and DMSO in which the rate of EA-inducing cells was ordinarily around 50 %. The cell viability was measured by the trypan blue-exclusion test.

Inhibitory Test of TPA-induced O_2^- Generation in Differentiated HL-60 Cells

Inhibitory test of TPA-induced O_2^- generation in DMSO-differentiated HL-60 cells was done as previously reported (Markert 1984, Murakami 1997). Briefly, to determine the inhibitory effect of O_2^- generation, the test compound dissolved in 5 μl of DMSO was added to DMSO-induced differentiated HL-60 cell suspension and incubated at 37°C for 15 min. The cells were washed with PBS twice for removal of extracellular test compound to omit O_2^- scavenging effect. TPA (100 nM) and cytochrome *c* solution (1 μg/ml) was added to the reaction mixture, which was incubated for another 15 min. The reaction was terminated by placing it on ice. After centrifugation at 250 g, the visible absorption at 550 nm was measured.

Inhibitory effects were expressed by a relative decreasing ratio of absorbance of test compounds to a control experiment.

Inhibitory Test of Intracellular Peroxide Formation in Differentiated HL-60 Cells

Intracellular peroxides were detected by DCFH-DA as an intracellular fluorescence probe (Bass 1983, Murakami 1997). DCFH-DA (50 μM) was added to differentiated HL-60 cell suspension which was incubated at 37°C for 15 min. After the test compound was added to the cell suspension, the mixture thus obtained was incubated at 37°C for 15 min, followed by stimulation with TPA (100 nM). After incubation for another 15 min, the reaction was stopped by addition of ethylenediaminetetraacetic acid, disodiumsalt dihydrate (EDTA) (10 mM). A flowcytometer (CytoACE 150, JASCO, Japan) was used to detect 2',7'-dichlorofluorescein (DCF) formed by the reaction of DCFH with intracellular peroxides. The TPA-treated cells showing fluorescence levels equal to (the means + 3 x standard deviations) those of the control cells or higher were regarded as "Peroxide Positive", and their rates were expressed as a "Peroxide Positive Rate (PPR)". Experimentation was repeated twice with similar results. The data are expressed by a representative histogram.

In Vitro *Antioxidative Assay*

An inhibitory test of lipid peroxidation was carried out using the ethyl linoleate autoxidation system (Nakamura 1996b, Osawa 1981). A DMSO solution (25 μl) of each sample was added to a solution mixture of ethyl linoleate (10 μl), Tween 20 (12.5 μl) and 50 mM phosphate buffer (5 ml, pH 7.0). The solution was incubated at 37°C in the dark for 3 days. Total TBARS formation was measured by the method previously reported (Ohkawa 1978).

O_2^- scavenging activity was measured by the XA/XOD system using SOD Test Wako® with some modifications (Ohnishi 1985). In this system, O_2^- scavenging activity is estimated by measuring the nitroblue tetrazolium (NBT) reduction and XOD inhibitory activities as follows. NBT reduction inhibitory activity and the XOD inhibitory activity were measured as previously reported (Murakami 1996b). O_2^- scavenging (SOS) activity is given by the following equation:

SOS activity (%) = NBT reduction inhibitory activity (%) - XOD inhibitory acitivity (%)

Chapter III:
Evidence for Suppression by ACA of Tumor Promoter-induced Oxidative Stress in Mouse Skin

Introduction

In Chapter II, the author demonstrated the inhibitory effects of ACA analogs on ROS formation as well as the structure-activity relationship. The positive correlation of the inhibitors of EBV activation between O_2^- generation inhibition and suppression of intracellular peroxide formation in differentiated HL-60 cells was elucidated. These results suggested that inhibition of O_2^- generation in leukocytes, at least in part, might be important for the suppression of oxidative stress and hence tumor promotion in mouse skin.

To prove this assumption directly, the author examines whether or not ACA suppresses TPA-induced oxidative stress in mouse skin using a double-application model. Specific inhibition of ACA in the activation phase of the inflammation process induced by double TPA treatments is clarified. Thus the hypothesis that inhibition of leukocyte activation should be significant is proposed. Further histological study clearly demonstrates the strong inhibition of oxidative damage in mouse skin through leukocyte regulation in inflammatory regions by ACA.

Results

Inhibitory Effects of ACA, HC, GEN and Allopurinol on TPA-induced H_2O_2 Production in Mouse Skin

HC is regarded as an appropriate analog for *in vivo* antioxidative studies since HC showed an opposite activity profile to ACA in tumor promoter-induced biological tests; EBV activation and O_2^- generation, and also in the lipid peroxidation test as described in Chapter II. As shown in Fig. III-1, double applications of 8.1 nmol TPA at 24-h interval increased the level of H_2O_2 by about 20-fold (7.01 ± 0.87 *versus* 0.36 ± 0.25 nmol / skin punch, $P < 0.001$) to that in the control mice treated twice with acetone instead of TPA. Pretreatment of ACA (810 nmol) before each TPA treatment almost completely inhibited H_2O_2 formation (0.79 ± 0.38 nmol / skin punch, IE = 89%). Genistein (GEN) at the same dose also significantly

reduced H_2O_2 formation (1.63 ± 1.27 nmol / skin punch, IE; 77%). Both compounds reduced the H_2O_2 level dose-dependently. On the other hand, HC at 810 nmol weakly inhibited H_2O_2 formation by 49% and showed no inhibitory effect at 81 nmol. Allopurinol, a well-known XOD inhibitor even at a dose of 10 μmol did not reduce the H_2O_2 level. ACA and HC have no effect on the level of externally added H_2O_2 *in vitro* (data not shown) while GEN was reported to be a H_2O_2 scavenger (Wei 1993b).

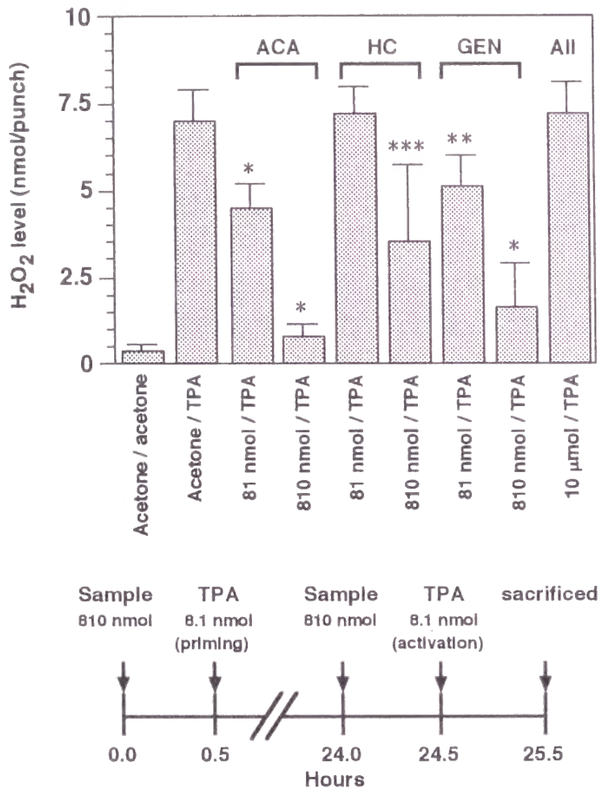

Fig. III-1 Inhibitory effects of ACA, HC, GEN and allopurinol on the H_2O_2 formation in mouse skin.
ICR mice were treated by a double treatment protocol as described here and in "**Experimental**". Mouse skins were treated with ACA, HC, GEN (81 and 810 nmol) or allopurinol (10 μmol) or acetone 30 min prior to each TPA treatment. The mice were sacrificed 1 h after the second TPA application, and their skins were removed for H_2O_2 assays. Significance was determined by the Student's *t*-test and is expressed as *, TPA *versus* acetone control, $P < 0.001$; **, Inhibitor/TPA *versus* TPA, $P < 0.001$; ***, Inhibitor/TPA *versus* TPA, $P < 0.05$.

Anti-inflammatory Activities of ACA, HC and GEN in Mouse Skin by Single TPA Application

The effects of ACA, HC and GEN on single application of TPA-induced inflammatory responses determined by skin edema formation and PMN infiltration, were examined. As shown in Table III-1, single TPA application (8.1 nmol) resulted in edema formation (as measured by the weight of skin punch) by 3.2-fold (99.8 ± 5.8 *versus* 31.2 ± 6.1 mg / skin punch, $P < 0.001$) and increasing in PMN infiltration (as measured by myeloperoxidase (MPO) activity) by 2.5-fold (6.99 ± 0.72 *versus* 2.80 ± 0.52 unit / skin punch, $P < 0.001$) as compared with the control. Pretreatment with neither ACA nor HC at 100-fold molar dose to TPA (810 nmol) reduced skin edema formation and PMN infiltration. GEN at 810 nmol significantly inhibited both biomarkers by 36% and 47%, respectively.

Table III-1 Inhibitory activities of ACA, HC and GEN (810 nmol) against inflammation induced by single dose of TPA in mouse skin.

	Edema (mg/punch)		MPO (units/punch)	
Treatment	m ± SD[a]	IE (%)	m ± SD	IE (%)
Acetone/acetone	31.2 ± 6.1	-	2.8 ± 0.5	-
Acetone/TPA	99.8 ± 5.8*[b]	-	7.0 ± 0.7*[b]	-
ACA/TPA	97.7 ± 6.8*[b]	3	6.2 ± 1.4*[b]	19
HC/TPA	99.2 ± 5.2*[b]	0.9	7.9 ± 0.8*[b]	-21
GEN/TPA	75.5 ± 2.6*[b, c]	36	5.0 ± 0.6*[b, c]	47

ICR mice (5 mice in each group) were treated as described in "**Experimental**". The mice were sacrificed 18 h after TPA treatment (8.1 nmol), and skin punches were obtained for determination of edema formation and MPO activity.
[a]Significance was determined by the Student's *t*-test and is expressed as *, $P < 0.001$.
[b]*versus* acetone/acetone control.
[c]*versus* acetone/TPA.

Inhibitory Effects of ACA, HC and GEN Applied in the Priming or Activation Phase on TPA-induced H_2O_2 Generation in Mouse Skin

To distinguish whether ACA, HC and GEN inhibit the priming or activation phase in a double TPA application model, each test compound was coadministered with either the first (priming) or second (activation) dose of TPA. Fig. III-2 shows the inhibitory effects of ACA, HC and GEN

applied prior to either first or second TPA treatment on H_2O_2 generation in skin. A dramatic decrease in the H_2O_2 level was observed in the mice to which ACA was coadministered in the activation phase (1.24 ± 0.29 nmol / skin punch, inhibitory effect; 87%). On the contrary, ACA applied in the priming phase exhibited no inhibition of H_2O_2 generation. HC showed no significant decrease in the H_2O_2 level in the priming phase and weak inhibition in the activation phase. GEN reduced the H_2O_2 level in both phases, and inhibition was remarkable in the priming phase rather than the activation phase (4.68 ± 1.43 *versus* 6.28 ± 1.43 nmol / skin punch).

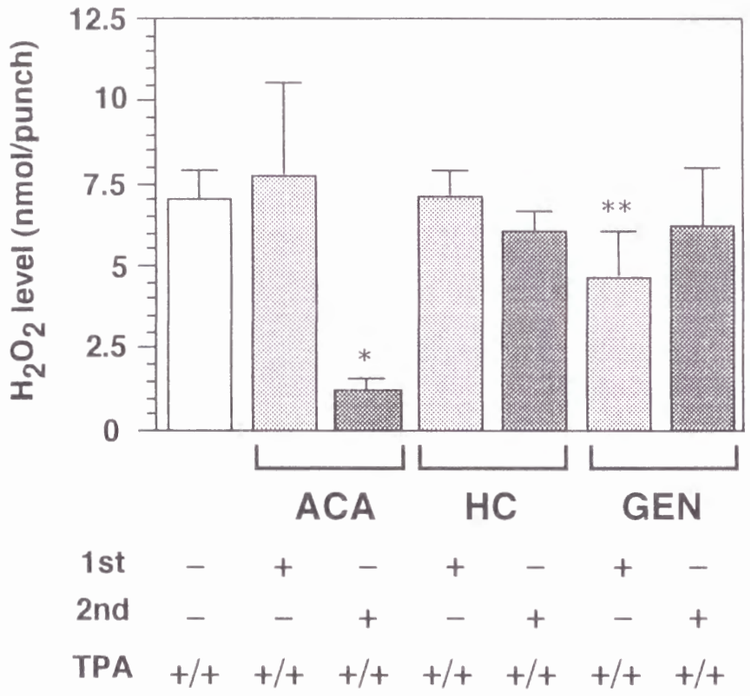

Fig. III-2 Inhibitory effects of ACA, HC and GEN applied in the priming or activation phase on the H_2O_2 formation in mouse skin.
Mice were treated with ACA, HC or GEN (810 nmol) or acetone 30 min before either 1st or 2nd TPA treatment. The mice were sacrificed 1 h after the second TPA application. Significance was determined by the Student's *t*-test and is expressed as *, Inhibitor/TPA *versus* TPA, $P < 0.001$; **, Inhibitor/TPA *versus* TPA, $P < 0.01$; ***, Inhibitor/TPA *versus* TPA, $P < 0.05$.

Inhibitory Effect of ACA on TPA-induced TBARS Formation in Mouse Epidermis

Because topically applied ACA afforded a significant inhibition against double TPA application-induced H_2O_2 generation, the author assessed whether or not such ACA treatment reduces double TPA application-induced TBARS formation, a well-known biomarker of overall oxidative damage to cellular constituents such as membrane lipids. The quantitative data for the level of TBARS formation in mouse epidermis homogenate detected in each group are shown in Fig. III-3. The increased level in TBARS caused by the single TPA application was highly significant to that of the control (0.52 ± 0.05 *versus* 0.36 ± 0.25 nmol / cm^2, $P < 0.01$). The net increase in TBARS level (control values subtracted) after the second application of TPA was 2.1-fold (0.76 ± 0.17 nmol / cm^2, $P < 0.05$) higher than that caused by the single TPA treatment. Pretreatment of ACA (810 nmol) before each TPA treatment inhibited the increase of the TBARS level by the second application of TPA (0.41 ± 0.14 nmol / cm^2, $P < 0.01$). The present data indicate that ACA significantly inhibits TBARS formation (Fig. III-3), known as a lipid peroxidation biomarker in mouse skin.

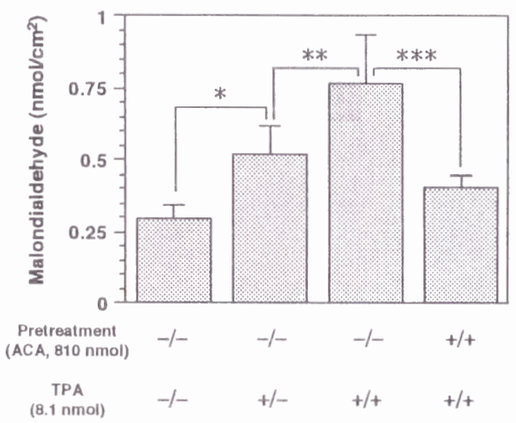

Fig. III-3 Inhibitory effects of ACA on the TBARS formation in mouse epidermis.
ICR mice were treated by the double treatment protocol as here and in "**Experimental**". Mouse skins were treated with ACA (810 nmol) or acetone 30 min prior to each TPA treatment. The mice were sacrificed 1 h after the second TPA application, and their epidermis removed for TBARS assays. The mice with single TPA were treated with TPA only in the priming phase. Significance was determined by the Student's *t*-test and is expressed as *, single TPA *versus* acetone control, $P < 0.01$; **, double TPA *versus* single TPA, $P < 0.05$; ***, double ACA/TPA *versus* double TPA, $P < 0.01$.

Effects of ACA on Morphological Changes in Mouse Skin by Double TPA Applications

It has been shown that double TPA treatments caused a dramatic increase in H_2O_2 levels. Thus this protocol was selected in a histological study to determine whether TPA application(s) to the skin enhances edema formation, leukocyte infiltration and hyperplasia. A single application of TPA induced obvious morphological change of inflammatory response (Table III-2, Fig. III-4*B*) as compared with the control group (Fig. III-4*A*), which was correlated to the results of skin edema formation and MPO activity (Table III-1). Mouse skin treated with TPA twice at a 24-h interval displayed severe epidermal hyperplasia (Fig. III-4*C*) and showed 2-fold increment in leukocyte infiltration as compared with that treated with a single TPA application (169 ± 43 *versus* 294 ± 27 / mm^2). A nuclear atypism of the epidermal layer and mitosis of epidermal cells in double TPA-treated mice became more remarkable than those with single TPA treatment (Fig. III-4*B* and *C*). On the other hand, pretreatment of ACA (810 nmol) before each TPA treatment diminished double TPA application-induced hyperplasia, mitosis and leukocyte infiltration, in which thinner epidermal layers and fewer leukocytes in the cutis were observed as compared with double TPA treated-mice (Fig. III-4*C* and *D*).

Table III-2 Inhibitory effect of ACA on double TPA treatment-induced morphological changes in mouse skin

Treatment[a]		Epidermal[b] thickness (μm)[c]	No. of epidermal layers[d]	No. of leukocytes in the cutis[e]
Pretreat	TPA (1st/2nd)			
Acetone	(−/−)	23.4 ± 2.9	2 (1-2)	8 ± 3
Acetone	(+/−)	32.1 ± 4.0[f]	4 (3-4)	169 ± 43[f]
Acetone	(+/+)	35.4 ± 4.5[f]	4 (2-5)	294 ± 27[f]
ACA X2	(+/+)	38.8 ± 3.7[f]	3 (2-4)	98 ± 44[f, g]

[a]Mices were treated as described in "**Experimental**". The mice were sacrificed 1 h after the second TPA treatment (8.1 nmol), and skin punches were obtained for determination of the morphological changes.
[b]Hyperplastic response of the skin determined in a section (5 μm) of the skin.
[c]Mean ± SD of 24 values taken from three individual mice.
[d]Majority (Range) of 24 values, counted in the various regions.
[e]Mean ± SD of 24 values, counted in the various regions of the cutis (dermis and subcutis) in a area of 1 mm^2 under microscope.
[f]Significant *versus* acetone and TPA (-/-) (Student's *t* -test); $P < 0.005$.
[f]Significant *versus* acetone and TPA (+/+) (Student's *t* -test); $P < 0.005$.

Fig. IV-4 Effect of ACA on TPA-induced mouse skin morphological changes. The protocol for animal treatment is as described in "**Experimental**". Treatment with: *A*, acetone; *B*, single dose of TPA in the priming phase; *C*, double dose of TPA; *D*, double dose of ACA and TPA. X40.

Discussion

Double applications of phorbol esters trigger ROS production in mouse skin (Kensler 1987, 1989). The available data reported previously suggested that each application induces a distinguishable biochemical event, namely, priming and activation (Ji 1992). The former is characterized as recruitment of inflammatory cells such as neutrophils by chemotactic factors to inflammatory regions and edema formation. The latter is the process of activation of neutrophils or other oxidants-producing cells including keratinocytes, in which the second TPA application of phorbol esters induces oxidative stress. Single application of TPA (8.1 nmol) significantly increased the weight of a skin punch as well as MPO activity, a biomarker for infiltration of the inflammatory cells into the target tissue (Table III-1). Double treatments of ICR mice with TPA (8.1 nmol) dramatically increased the formation of H_2O_2 in the skin (Fig. III-1) as previously reported using outbred species of mice such as SENCAR or CD-1 (Wei 1992, 1993a,b). Importantly, the TPA dose used in the present experiment is in the tumor promotion range (1-10 nmol). The requirement of second TPA treatment for ROS production was pointed out by Ji *et al.* and confirmed in the present experiments in which a single dose application of TPA did not significantly enhance H_2O_2 production 1~24 h after treatment (1h and 18 h after application; 0.30 ± 0.18 nmol / skin punch and 0.50 ± 0.28 nmol / skin punch, respectively) as compared with the control treatment (0.36 ± 0.25 nmol / skin punch).

Evidence for the involvement of ROS production by double or multiple TPA treatments in carcinogenesis have been presented. Wei *et al.* (1991, 1992, 1993a) found the increased levels in oxidized DNA bases in mice with double TPA treatment and with multiple TPA treatment for 16 weeks. The positive correlation among the formation of H_2O_2, oxidized DNA bases and first-stage tumor-promoting activity has been designated (Wei 1991). It is well-known that O_2^- is converted to H_2O_2 non-enzymatically or by the function of superoxide dismutase (SOD) in biological systems. The hydroxyl radical, formed subsequently from H_2O_2, randomly reacts with biological components within cell. Recently Takeuchi *et al.* (1996) reported that hydroxyl radicals may directly induce formation of 8-hydroxydeoxyguanosine in DMSO-differentiated HL-60 cells. Alternatively, hydroxyl radicals react with membrane lipids to form

hydroperoxides which are then decomposed and converted to mutagenic, reactive carbonyl compounds such as malondialdehyde. On the other hand, the oxidative metabolic activation of proximate carcinogens, independent on the cytochrome P-450 pathway, has been proven to be associated with multiple treatments of mice with a tumor promoter. Pound (1967) reported that treatment of mice with a tumor promoter prior to that of a tumor initiator enhanced the initiation process. It has been presumed that not only the stimulation of DNA synthesis but also remodeling of the epidermal metabolic pathway from the cytochrome P450-dependent pathway to an oxygen radical-dependent pathway increases the probability of mutation (Ji 1992). It should be noted that MPO was involved in the bioactivation of a procarcinogen in mouse skin and lung (Kensler 1987, 1989, Petruska 1992, Trush 1994).

The present results provide clear evidence for the suppression of tumor promoter-induced H_2O_2 formation in mouse skin by ACA. It is noteworthy that ACA exhibited no inhibitory effects on edema formation and the enhancement of MPO activity in the first TPA treatment while a radical scavenger GEN administered at the same dose (810 nmol) significantly suppressed these inflammatory biomarkers (Table III-1). ACA at 810 nmol also did not inhibit TPA (8.1 nmol) -induced mouse ear edema formation 6 h after treatment (data not shown). No inhibitory potential of ACA for the TPA-induced priming phase was clearly confirmed by the experiment in which ACA was coadministered only with the first dose of TPA (Fig. III-2). It is likely that induction of lipoxygenase, which metabolizes arachidonic acid to hydroperoxy fatty acids, precursors of chemotactic leukotrienes, represents the earlier stage in the priming phase. Conversely, ACA showed no inhibitory effects on TPA-induced arachidonate release or prostaglandin E_2 synthesis in HeLa cells (data not shown). Taken together, the inhibition of some biological events in the priming phase by ACA can be ruled out as the critical mechanism of H_2O_2 generation inhibition *in vivo*.

Coadministrations of ACA only with the second TPA treatment successfully inhibited H_2O_2 formation whereas HC and GEN in the same treatment failed to do so (Fig. III-2). *In vitro*, ACA exhibited potent inhibition of O_2^- generation (IC_{50} = 4.3 μM). On the contrary, the inhibitory activities of HC (> 100 μM) and GEN (102 μM, Murakami 1996b) were

much lower than that of ACA. Thus, there is a positive correlation between inhibition of the activation phase *in vivo* and suppression of leukocyte activation *in vitro*. A well-known XOD inhibitor, allopurinol also showed no inhibitory effect on double TPA treatment-induced H_2O_2 generation. These findings appeared to be consistent with a mechanism in which the NADPH oxidase system of neutrophils rather than the epithelial XOD system is implicated in the O_2^- generating system in double TPA-treated mouse skin. Single-dose TPA-induced enhancement of XOD activity was reported (Reiners 1987) but in the present study single-dose TPA did not significantly enhance the H_2O_2 formation, suggesting the critical role of NADPH oxidase system in double TPA application-induced oxidative stress. Single TPA application enhances oxidized DNA base formation much less than double application of TPA (Wei 1993a), also supporting this suggestion. Conversely, the flowcytometric studies on isolated epidermal cells by Robertson *et al*. (1990) demonstrated that single TPA application resulted in increases in both keratinocytes and neutrophils. It should be noted that in their study subpopulations of keratinocytes, characterized by an increased density within cells as compared with the major population in the same epidermal, were present in single TPA-treated mouse epidermis and produced much greater amounts of H_2O_2 than the major populations. Thus the exact cell type(s) for the source of dramatic production of H_2O_2 by double TPA treatment need to be identified. The possibility of up-regulation of glutathione levels which decompose H_2O_2 may be ruled out in this mechanism since ACA showed no influence on the total glutathione levels and significantly decreased oxidized glutathione levels in double TPA applications (data not shown).

The present data indicate that ACA, a weak antioxidant in lipid peroxidation *in vitro* (Table II-4), significantly inhibits TBARS formation (Fig. III-3), known as an overall oxidative damage biomarker, which was, at least in part, formed downstream of H_2O_2 generation in the presence of a metal ion as catalyst. TBARS formation *in vivo* is considered not to reflect a single particular phenomenon but to indicate widespread oxidative damage including lipid peroxidation, mitochondrial deenergization and protein or sugar degradation rather than DNA (Nakae 1994). Cell death and subsequent regenerative cell proliferation, which gives growth advantage to phenotypically altered populations, are accepted to play critical roles in

some organ carcinogenesis in rodents (Farber 1990). The inhibitory effect of ACA on double TPA application-induced morphological changes in mouse skin, such as mitosis of epidermal cells (Fig. III-4), suggested that suppression of oxidative damage through ROS generation inhibition is an important action mechanism of anti-tumor promotion.

Histological studies demonstrated very clearly that double TPA treatment enhances morphological changes reflecting inflammatory response. A single application of TPA induces edema formation and leukocyte infiltration similarly to the result of biochemical detection (Table III-1). Surprisingly, a significant increase (1.7-fold) in leukocyte infiltration was observed 1 h after the second TPA application (Table III-2). It should be noted that, although inhibiting neither the arachidonate metabolism nor single application-induced edema formation, ACA could reduce the increment in numbers of leukocytes in mouse cutis. Kensler *et al*., (1989) presumed that the second TPA could additionally recruit leukocytes, which are phenotypically different from those recruited by a single TPA treatment. In this respect, it is important to note that O_2^- is known to participate in the formation of chemotactic factors and recruitment of PMNs (Warren 1990). Thus, it is tempting to recognize that suppression of leukocyte infiltration by ACA, which was observed in the histological study, is attributable to the inhibition of the second dose of TPA-induced O_2^- generation by leukocytes. In any case, the author concluded that ACA potently suppresses TPA-induced oxidative stress mainly via regulation of leukocytes including inhibition of ROS generation and interfering with their infiltration to the inflammatory regions of the epidermis and dermis, though the latter was confined only by histological study. Sisskin *et al*. (1982) pointed out that the result of a single topical application of TPA might not predict the response with multiple treatments. Conversely, the present study strongly suggests that the double TPA application experiment is appropriate not only to predict the inhibitory potential of test compounds for chronic inflammation or tumor promotion but also to address their action mechanisms.

Interestingly, ACA exhibited chemopreventive effects on chemically-induced carcinogeneses in rat colon and tongue, where acute and/or chronic inflammation are considered to be closely associated with it. In a rat oral carcinogenesis model, inflammatory cell infiltration is predominant in macrophage, which is related to immune function alteration,

5-6 months after oral carcinogenic 4-NQO treatment (Thomas 1995). It is well known that chronic inflammation of the colon is associated with an increased risk of colorectal cancer. Recently, neutrophil-mediated nitrosamine formation has been indicated to be a possible endogenous carcinogen which may promote neoplasia (Grisham 1992). Suppressive effects of ACA on leukocyte-derived ROS formation may be, at least in part, involved in a common mechanism for the inhibition of inflammation-related carcinogenesis.

In the present study, the author demonstrated that ACA, acting as a O_2^- generation inhibitor, was of greater advantage for suppression of *in vivo* ROS generation than HC, having strong antioxidative activity in the lipid peroxidation system but not in the O_2^- generation system in differentiated HL-60 cells. Recently an extremely interesting study on the evaluation of the sensitivity of transgenic mice with over expression of GPx or both GPx and SOD to skin tumor promotion has been described by Lu *et al.* (1997). Surprisingly, these transgenic mice had *enhanced* tumorigenic response to application of DMBA/TPA. The mechanism of this phenomenon is not yet well-understood. They, however, have presumed that the altered ROS-detoxification enzyme levels might influence the process of carcinogenesis by modulating cell growth phenotype, increasing resistance of cells with oxidative damage, or by altering immune function. These results encounter the difficulty in regulation of the ROS level and for prevention of cancer by radical-scavenging type antioxidants. In this regard, ACA, a ROS generation inhibitor, may be evaluated as a unique and effective chemopreventive agent because one does not need to regard the ROS level when using ACA in rodents models. Studies on the influences of ACA on immune function such as natural killer activity or the T cell-mediated immune system as well as investigation of ACA in a wide range of animal carcinogenesis models relating to inflammation are necessary.

Experimental

Chemicals

An authentic malondialdehyde (1,1,3,3-tetramethoxypropane) was purchased from OXIS International Inc., OR, USA. All of the other chemicals were obtained as described in Chapter II.

Treatment of Animals

Female ICR mice (7 weeks old) were obtained from Japan SLC, Shizuoka, Japan. Mice used in each experiment were supplied with fresh tap water *ad libitum* and rodent pellets (MF, Oriental Yeast Co., Kyoto, Japan) freshly changed twice a week. Animals were maintained in a room controlled at 24 ± 2°C with a relative humidity of 60 ± 5 % and a 12-h light/dark cycle (06:00 to 18:00). The back of each mouse was shaved with surgical clippers two days before each experiment. All test compounds (100 µl in acetone) were topically applied to the shaved area of the dorsal skin 30 min before application of a TPA solution (8.1 nmol/100 µl in acetone). In the double-treatment protocol, two same doses of TPA and test compounds or acetone were applied at an interval of 24 h.

Anti-inflammation Test in Mouse Skin

Two biomarkers of skin inflammation, edema formation and MPO activity, were determined by the method of Wei *et al.* (1992) with slight modifications. Mice were sacrificed by cervical dislocation 18 h after a single application of TPA. The mouse skin punches were obtained with an 8-mm-diam cork borer and weighted in an analytical balance. The inhibitory effects (IE) were expressed by the relative increasing ratio of the weight of a treated punch to that of a control punch; IE (%) = [(TPA alone) - (test compound plus TPA)] / [(TPA) - (vehicle)] x 100. Statistical analysis was done by the Student's *t*-test. For the determination of MPO activity, the skin punches were minced in 3 ml of 0.5% hexadecyltrimethyl ammonium bromide in 50 mM potassium phosphate buffer, pH 6.0 and homogenized at 4°C for 10 s twice. Samples were centrifuged at 10,000 g for 20 min at 4°C. To each 2-ml cuvette, 0.65 ml of 25 mM 4-aminoantipyrine-2% phenol solution and 0.75 ml of 2 mM H_2O_2 were added to equilibrate for 5 min. After the basal rate was established, a 100-µl sample supernatant was added to the cuvette and quickly mixed. Increases in absorption at 510 nm for 1 min

at 0.1-min intervals were recorded. The MPO activity was calculated from the linear portion of the curve and expressed as units of MPO per skin punch. One unit of MPO activity is defined as the activity that degrades 1 μmol of H_2O_2 per min at 25°C.

Determination of H_2O_2 in Mouse Skin

Mice treated by a double-treatment protocol were sacrificed 1 h after the second TPA treatment. Their skin were removed and then immersed in a 55°C water bath for 30 s, and the subcutis was scraped off. The skin (epidermis and dermis) punches were obtained with 8-mm-diam cork borer and weighed by an analytical balance. The skin punches were minced in 3 ml of 50 mM phosphate buffer (pH 7.4) containing 5 mM sodium azide and then homogenized at 4°C for 30 s twice. The homogenate was centrifuged at 10,000 g for 20 min at 4°C. The H_2O_2 content was determined by the phenol red-horseradish peroxidase (HRPO) method (Wei 1992, 1993b). To each 1.5-ml cuvette, 0.5 ml of the homogenate supernatant and 0.5 ml of the phenol red (200 μg/ml)-HRPO (100 μg/ml) solution were added and incubated at 25°C for 10 min. At the end of the incubation, 100 μl of 1M NaOH was added to terminate the reaction, and then absorbance was determined spectrophotometrically at 610 nm. The final results were expressed as equivalents of nanomoles of H_2O_2 per skin punch, on the basis of a standard curve of HRPO-mediated oxidation of phenol red by H_2O_2.

Determination of TBARS in Mouse Epidermis

Mice treated with a double-treatment protocol were sacrificed 1 h after the second TPA treatment. Skin samples from treated areas were excised and immediately dipped into ice-cold PBS. After subcutis were scrapped off, the skin was cut into small species, and floated, epidermis side down, in a culture dish with a 2.5% trypsin solution at 37°C for 45 min. The epidermis was then scrapped off and put into a cuvette with 450 μl of 50 mM phosphate buffer (pH 7.4). Fifty μl of 50% trichloroacetic acid solution was added and sonicated for 10 min. After freeze-thawed five times, the sample was centrifuged for 10 min at 10,000 g. The TBARS level of mouse epidermis was determined by the method described in Chapter II. The final results were expressed as equivalents of nanomoles of malondialdehyde per

cm^2, on the basis of a standard curve of TBARS formation using an authentic malondialdehyde.

Histological Study of Hyperplasia and Leukocyte Infiltration

Mice treated with a double-treatment protocol were sacrificed 1 h after the second TPA treatment. Excised skin was fixed in 10% formalin, and then embedded in paraffin. Vertical sections (5 μm) were cut, mounted on a glass slide, and stained with hematoxylin and eosin. For each section of skin, the thickness of the epidermis from the basal layer to the stratum corneum was measured at five equidistant interfollicular sites utilizing Nikon YS2, Alphaphot-2 (Japan) equipped with an occular micrometer as previously reported (Katiyar). The number of infiltrating leukocytes were counted at least at eight different sections in each group under the same microscope by utilizing marked grid.

Chapter IV:
Classification of Chemopreventive Food Phytochemicals by Their Antioxidative Profiles in Mouse Skin

Introduction

The author has regarded inhibition of tumor promotion (anti-tumor promotion) with food phytochemicals to be one of the best strategies for chemoprevention since tumor promotion, taking a long-time to occur, is a reversible stage in multistage carcinogenesis (Pitot 1991). Such characteristics implicate the efficiency of anti-tumor promotion for cancer control in humans. So far, anti-tumor promoting properties of edible Japanese plants (Koshimizu 1988), marine algae (Ohigashi 1992), and edible plants in southeast Asia (Murakami 1993, 1995a, 1998b) have been extensively estimated by a Japanese group including the author. In the continuous search for useful chemopreventive agents from edible plants, some constituents have been isolated and identified as EBV activation inhibitors (Fig. IV-1), and then proven to be inhibitors of TPA-induced tumor promotion in mouse skin as shown in Table IV-1.

In this chapter, the author assesses inhibitory effects of several chemopreventive agents, the chemical and biochemical characteristics of which are shown below, on TPA-induced H_2O_2 production in mouse skin to clarify which event caused by TPA in inflammation processes, priming and/or activation, is inhibitable by such agents. Further mechanistic studies using *in vitro* assays of ROS generation and inflammation processes are conducted to demonstrate their regulation of inflammatory leukocytes for the inhibition of double TPA treatment-induced oxidative damage in mouse skin.

ACA

Isolation and identification of ACA as a chemopreventer are described in Chapters II and III.

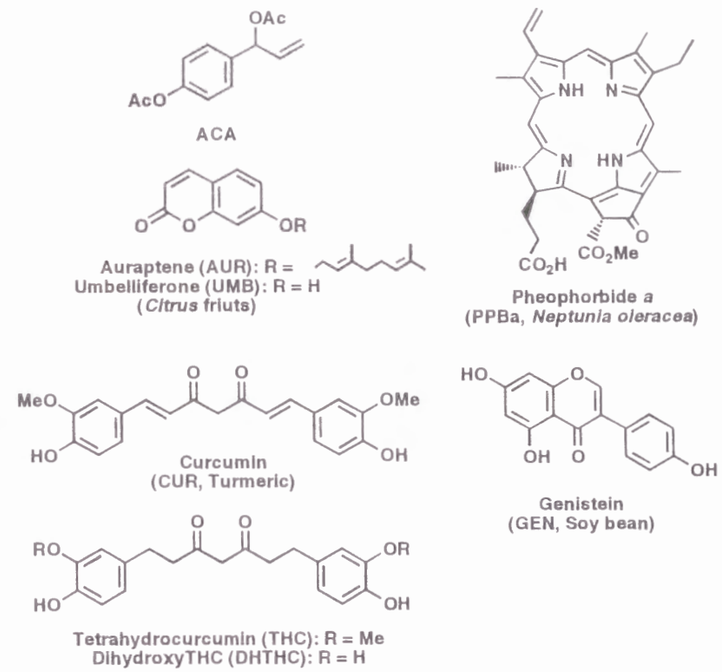

Fig. IV-1 Chemical structures of chemopreventers form edible plants

Table IV-1 Anti-tumor promoting activities of food phytochemicals in mouse skin

Compound	(nmol)	% Inhibition of No. of tumors/mouse	Ref.
ACA	1.6	44	Murakami, 1996a
	160	90	
AUR	16	19	Murakami, 1997
	160	23	
PPBa	16	44	Nakamura, 1996b
	160	56	
CUR	100	69	Huang, 1997
THC	1000	42	Huang, 1995
GEN	1000	36	Wei, 1995

Auraptene

Citrus fruits are widely known to contain a variety of chemopreventive agents, e.g., limonoids and their glucosides as inhibitors of benzo[*a*]pyrene-induced forestomach and lung carcinogenesis in mice, TPA-induced skin tumor promotion in mice (Lam 1994), DMBA-induced oral carcinogenesis in hamsters (Miller 1994), *d*-limonene as an inhibitor of azoxymethane-induced rat colonic ACF formation (Kawamori 1996), DMBA-induced rat mammary carcinogenesis (Crowell 1992), and flavonoids (Le Bon 1993) such as hesperidine as inhibitors of 4-NQO-induced rat oral carcinogenesis (Tanaka 1994) and azoxymethane-induced rat colonic ACF formation (Tanaka 1996). The author *et al.* have recently reported that a glyceroglycolipid from the leaves of bitter orange (*Citrus hystrix*) possess strong inhibitory effects on TPA-induced skin tumor promotion (Murakami 1995b).

Recently, auraptene (AUR), a coumarin-related compound, have been isolated from the cold-pressed oil of *natsumikan* (*Citrus natsudaidai* HAYATA), as an inhibitor of the tumor promoter TPA-induced EBV activation in Raji cells. The 50% inhibitory concentration (IC_{50}) of AUR (18 μM) was equal to that of genistein (GEN, IC_{50} = 19 μM), while interestingly, umbelliferone (UMB) lacking a geranyloxyl group in AUR was inactive (IC_{50} = 450 μM). In a two-stage carcinogenesis experiment with TPA (topical application at 1.6 nmol) and DMBA (0.19 μmol) in ICR mouse skin, topical application of AUR (at 160 nmol) significantly reduced tumor incidence and the number of tumors per mouse by 27% ($P < 0.01$) and 23% ($P < 0.05$), respectively (Table IV-1, Murakami 1997). The inhibitory effect of 4-NQO-induced rat tongue carcinogenesis (Tanaka 1998) and azoxymethane-induced colonic ACF formation (Tanaka 1997c) have also been reported.

Pheophorbide a

Chlorophyll-related compounds have been shown to exert antimutagenic and anticarcinogenic behavior against a wide range of potential human carcinogens (Hayatsu 1993). Inhibitory effects of chlorophyllin, a water-soluble derivative of chlorophyll, on *in vivo* carcinogenesis have recently been reported (Breinholt1995, Guo 1995a,b, Hasegawa 1995, Park 1995, 1996, Singh 1996). Thus, chlorophyll-related

compounds are expected to be one of the most useful chemopreventive agents because of their relative abundance in green plants. However, there are few studies on anti-tumorigenesis activity of chlorophyll-related compounds other than chlorophyllin.

Pheophorbide *a* (PPBa) is known to be one of the significant degradation products of chlorophyll *a*, and is formed by the removal of a magnesium ion and a phytyl group through the action of chlorophyllase in plants or bacteria under an acidic condition (Strain 1966). In the previous study, the author isolated PPBa, from the leaves of *Neptunia oleracea*, an aquatic perennial herb called "water mimosa" that is used as a sour vegetable in Thailand, found it to be an effective inhibitor (IC_{50} = 3.3 μM) of EBV activation (Nakamura 1996a). PPBa is known as a dietary photosensitizer (Endo 1982, Kawabata 1989). This raised the possibility that photo-induced oxidative damage resulted in EBV activation inhibition. As a preliminary approach to explore the inhibitory mode of PPBa, the author examined the enhancing effect of the photosensitization of PPBa toward EBV activation inhibition. PPBa showed no significant difference in inhibitory activity with or without irradiation, suggesting that the photosensitizing effect of PPBa was not involved in the major mode of EBV activation inhibition (Nakamura 1996a).

As shown in Table IV-1, the author first confirmed the *in vivo* anti-tumor promoting activity of PPBa. Topical application of PPBa even at a 10-fold dose to TPA (16 nmol) significantly reduced the average number of tumors per mouse. This activity was comparable to those of anti-tumor promoters from edible plants such as glyceroglycolipids (Murakami 1995b), quercetin (Nishino 1984a), and glycyrrhetic acid (Nishino 1984b). Topical application of PPBa showed no tumor-promoting effect on mouse skin carcinogenesis up to a dose of 160 nmol. Although PPBa, given a high dose, caused photo-induced oxidative damage to the cell membrane by generation of singlet oxygen ($^1O_2$) (Kawabata 1989), it is important to note that the topical application of PPBa to mouse skin resulted in no marked damage or injury under normal light conditions.

Curcuminoids

Curcumin (CUR, diferuloylmethane), a major yellow pigment of turmeric occurring in the rhizomes of several tropical gingers such as

Curcuma longa, is commonly used as a coloring condiment. A wide range of biological and pharmacological activities of CUR have thus far been investigated (Govindarajan 1980, Huang 1992b). CUR is a potent inhibitor of mutagenesis and chemically induced carcinogenesis (Azuine 1992, Huang 1988, 1992c, 1994, 1995, 1997a,b, Nishino, 1987, Rao 1995, Tanaka 1994). For example, a topical application of commercial food grade CUR containing its minor derivatives or pure CUR strongly inhibited DMBA-induced tumor initiation and TPA-induced tumor promotion in mouse skin (Huang 1988, 1992c, 1995, 1997a,b, Nishino 1987). Dietary CUR also inhibited chemical carcinogenesis in a wide range of the target organs of rats and mice (Azuine 1992, Huang 1994, Rao 1995, Tanaka 1994). Thus, CUR is currently attracting wide attention because of its relatively low toxicity to rodents. Recently the author identified CUR as an EBV activation inhibitor from a tropical ginger *Zingiber cassumunar*.

The action mechanism(s) of CUR for its anti-tumor promoting activity is complicated. CUR inhibited TPA-induced skin inflammation (Huang 1992d) and also showed various biological activities relating to anti-tumor promotion, e.g., inhibition of DNA synthesis (Huang 1988, 1997b), cellular protooncogene expression such as c-*fos*, c-*jun*, and c-*myc*, (Kakar 1994) transcriptional factor AP-1 activation (Huang 1991), ornithine decarboxylase activity (Huang 1988) and PKC activity (Liu 1993). Curcuminoids exhibited antioxidative activities in some *in vitro* lipid peroxidation systems (Osawa 1995, Sharma 1976, Sugiyama 1996) and suppressed TPA-induced H_2O_2 production and oxidized DNA formation in mouse epidermis (Huang 1997b). CUR is also an inhibitor of neutrophil responses (Srivastava 1989) or of O_2^- generation in macrophages (Joe 1994).

In recent studies, tetrahydrocurcumin (THC, Fig. IV-1), one of the major colorless metabolites of CUR in a form of the glucuronide conjugate in the bile, exhibited stronger antioxidative activity than CUR in several *in vitro* systems (Osawa 1995, Sugiyama 1996). Thus THC was thought to be one of the metabolites with higher physiological and pharmacological activities than CUR in the intestine. Although these strong antioxidative actions of curcuminoids have been regarded to play some important roles in their anti-tumor promotion, there are few studies of the inhibitory effects of curcuminoids on *in vivo* oxidative stress. On the other hand, THC, a stronger *in vitro* antioxidant than CUR, has recently been reported to be a less

effective chemopreventive agent in mouse skin than CUR (Table IV-1). In contrast to the result of skin carcinogenesis, inclusion of 0.5% THC in the diet significantly inhibited 1,2-dimethylhydrazine-induced mouse colon carcinogenesis while the inhibitory effect of CUR was not statistically significant (Kim 1996). THC and dihydroxytetrahydrocurcumin (DHTHC) having *ortho* diphenol moieties showed less inhibitory activity against EBV activation than CUR (data not shown).

Genistein

Epidemiological studies show that consumption of soybean-containing diets have been associated with lower incidence of certain human cancers in Asian populations when compared to Caucasian populations (Setchell 1984). Animal experiments suggested that soybean diets inhibit radiation- and chemical-induced tumors of mamma (Barnes 1990, Troll 1980), skin (Troll 1970), and liver (Becher 1981). Although several studies attributed the anticarcinogenic effect of soybeans to protease inhibitors, a study conducted by Barnes *et al.* (1990) showed that alter inactivation of protease inhibitors by autoclaving, soybean diets still strongly inhibited carcinogen-induced mammary tumors in rats. This observation led to the hypothesis that soybean isoflavones may be responsible for the anticarcinogenic effect of soybean. Genistein (GEN) is the major isoflavone in soy and has been identified as a potent inhibitor of protein tyrosine kinases *in vitro* (Akiyama 1987). Since tyrosine phosphorylation plays a crucial role in cell proliferation and transformation, GEN may have important anticancer properties. Recently the inhibitory effect of GEN on tumor promoter-induced EBV activation was confirmed (Murakami 1997). The inhibitory activity of GEN against mouse skin and mammary carcinogenesis have been reported (Constantinou 1996, Lamartiniere 1995a,b, Wei 1995). The action mechanisms of GEN for its anti-tumor promotion in mouse skin have been postulated to be its anti-inflammatory action, inhibition of both ROS generation and protooncogene products expression (Wei 1993, 1995).

Results and Discussion

Inhibitory effects of the chemopreventive food phytochemicals on double TPA treatment-induced H_2O_2 are summarized in Table IV-2. Each total inhibition was determined by the mice pretreated with the test compound before each TPA treatment. To distinguish whether they inhibit the priming or activation phase in a double TPA application model, experiments were conducted in such ways that the test compounds were coadministered with either the first (priming) or second (activation) dose of TPA. Inhibitory effects on single TPA application-induced edema formation and O_2^- generation in differentiated HL-60 cells were determined to clarify whether or not these compounds exhibit the decreasing level of leukocyte infiltration and the suppression of leukocyte activation (Table IV-3).

Table IV-2 Inhibitory effects of chemopreventers from edible plants on TPA-induced H_2O_2 production in mouse skin

Compound (810 nmol)	Total inhibition	Inhibitory effect in priming phase	Inhibitory effect in activation phase
ACA	+++	-	+++
HC	+	-	-
GEN	+++	+	-
CUR	++	+	+
THC	+	N.T.*	N.T.*
DHTHC	+	N.T.*	N.T.*
PPBa	++	++	-
AUR	+++	-	+++
UNB	-	-	-

+++: Inhibitory effect (IE) ≥ 70%, ++: 70% > IE ≥ 50%, +: 50% > IE ≥ 20 %, -: 20% > IE.
*Not tested.

As shown in Table IV-2, the total inhibitory effects of ACA, GEN, CUR, PPBa, and AUR were significant (IE>50%) while inactive (UMB) or weakly active derivatives (THC and DHTHC) in EBV assays showed less inhibitory effect as compared with AUR and CUR, respectively. The specificity of these chemopreventers for inhibition in the priming or activation phase can be classified as follows.

Table IV-3 Inhibitory effects of chemopreventers on TPA-induced edema formation in mouse skin and O_2^- generation in differentiated HL-60 cells

Compound	Inhibition of edema formation (810 nmol, %)	Inhibition of O_2^- generation (IC_{50}, μM)
CUR	38	12
THC	20	29
DHTHC	18	15
PPBa	75	50
AUR	N.T.	1.2
UNB	N.T.	290

Activation Phase-specific Inhibitors (ACA and AUR)

Similarly to ACA, the specific inhibition of AUR in the activation phase was observed (Table IV-2), while UMB showed no effect in the same phase. It should be noted that the IC_{50} value for O_2^- generation inhibition of AUR (1.2 μM) was extremely lower than those of UMB (290 μM) and GEN (102 μM), and slightly lower than that of ACA (IC_{50} = 4.3 μM), as described in the previous chapter. On the other hand, preliminary study demonstrated that AUR showed no effect on single TPA application-induced mouse ear edema formation (data not shown). Moreover, similarly to ACA, AUR failed to scavenge O_2^- radicals in the XOD system (data not shown). A positive correlation of the structure-activity relationships of AUR and UMB among inhibition of O_2^- generation in leukocytes, total inhibition of H_2O_2 production in mouse skin and inhibition in the activation phase suggested that AUR was characterized as a highly selective O_2^- generation inhibitor in leukocytes but was not a radical scavenger. Thus it is likely that AUR may effectively suppress TPA-induced oxidative stress mainly by inhibition of O_2^- generation in leukocytes, possibly playing the major role for its anti-tumor promotion.

Priming Phase-specific Inhibitors (PPBa and GEN)

PPBa specifically inhibited H_2O_2 production when administered in the priming phase. PPBa strongly inhibited TPA-induced edema formation in mouse skin while the inhibitory effect of PPBa on O_2^- generation in

differentiated HL-60 cells was much weaker than that of CUR or AUR (Table IV-3). It is interesting to note that some phospholipase A_2 (PLA_2) inhibitors have suppressed O_2^- generation in mouse skin (Yoon 1993) and that they showed an anti-carcinogenic effect in the rat mammary gland (McCormick 1988), suggesting that PLA_2 is one of the key enzymes linking inflammation with carcinogenesis. Though not inhibiting type II PLA_2 from *Crotalusatrox* Venom even at a concentration of 100 μM, PPBa significantly inhibited soybean lipoxygenase (SBL) at the same concentration (Inhibition rate = 30%, unpublished data). SBL has homology in the amino acid sequence of the active site with human 5-lipoxygenase (5-LO) (Funk 1987, Shibata 1987). 5-LO catalyzes the first step in the metabolic pathway of arachidonic acid, which results in the formation of leukotrienes activating phagocytic leukocytes. Moreover, it was reported that some 5-LO inhibitors effectively inhibited skin tumor promotion (Jiang 1994, Yamamoto 1992). From this point of view, the inhibitory nature of PPBa against arachidonate metabolism-related enzymes including 5-LO may play an important role in its oxidative damage inhibition and hence anti-tumor promotion in mouse skin. Conversely, on the basis of the result of no inhibitory effect of PPBa in the activation phase of double TPA treatment-induced H_2O_2 production, inhibition of O_2^- generation in leukocytes was ruled out from the critical mechanism of oxidative damage inhibition in mouse skin.

On the other hand, the antioxidative activity of chlorophyll-related compounds in the dark have been reported (Cahyana 1992, Endo 1985, Sakata 1990, Sato 1986). The author also pointed out that PPBa showed antioxidative activity against lipid autoxidation in the dark, which was comparable to that of α-tocopherol (Nakamura 1996b). This finding suggested that direct inhibition by PPBa against the non-enzymatic formation of peroxides or reactive carbonyl compounds might, at least in part, participate in the antioxidative mechanism of PPBa in mouse skin.

Non-phase-specific Inhibitors (CUR and Its Derivatives)

A decrease in H_2O_2 level was observed in both groups of mice to which CUR was coadministered in the priming and activation phase. It is likely that THC and DHTHC, the derivatives of CUR, inhibit both phases. CUR has been reported to be an inhibitor of neutrophil responses (Srivastava 1989), generation of O_2^- in macrophages (Joe 1994) and a

scavenger of active oxygen radicals (Kunchandy 1990). The present study also demonstrated that CUR significantly inhibited TPA-induced O_2^- generation in differentiated HL-60 cells (Table IV-3). In the present system of O_2^- generation, the extracellular O_2^- scavenging effect of a test compound can be neglected because the test compound is washed out from the system by washing before TPA stimulation. NADPH oxidase is known to play a major role in O_2^- generation in leukocytes including macrophages, neutrophils, or granulocytes (Cross 1991). The multicomponent NADPH oxidase system consists of heterodimeric cytochrome *b* consisting of the β-subunit (gp91-*phox*) and α-subunit (p22-*phox*) associated with p47-*phox* and p67-*phox* (Henderson 1996). A direct role of PKC or PLA_2 for the activation of the assembled NADPH oxidase in neutrophil has been suggested (Curnutte 1994, Dana 1994). Thus, CUR may inhibit TPA-induced assembly of this NADPH oxidase system or upstream signal transduction systems possibly by inhibition of PKC activation (Liu 1993). CUR and THCs exhibited much stronger inhibition against O_2^- generation than phenolic radical scavengers such as rosmarinic acid, caffeic acid and ferulic acid (data not shown). In addition, the IC_{50} values of CUR (12 μM) and THCs (15-29 μM) against O_2^- generation were much lower than that of GEN (IC_{50} > 100 μM). On the other hand, the inhibitory effect of THC on O_2^- generation was weaker than that of CUR, while DHTHC, having two *ortho* diphenol moieties, significantly inhibited O_2^- generation comparable to CUR. These results suggested that the conjugated double bonds of the central seven carbon chain and the *ortho* diphenol moieties are important for enhancing the inhibitory effect of O_2^- generation in differentiated HL-60 cells. Such trends in the structure-activity relationship of the curcuminoids were distinct from those in lipid peroxidation tests as reported previously (Osawa 1995, Sugiyama 1996), but were parallel to inhibition of *in vitro* lipoxygenase or tyrosinase activity (Osawa *et al.* unpublished data). It is likely that the conjugated double bonds of the central seven carbon chain of CUR may enhance the metal ion chelating ability of the β-diketone moiety. These observations suggested that metal cheletion of the curcuminoids may play a critical role for the inhibition of these metalloenzymes, but may not be sufficient for antioxidation against propagation of lipid peroxidation.

The present results also demonstrate that the curcuminoids inhibit TPA-induced edema formation in mouse skin. CUR is a well-known

inhibitor of arachidonic acid metabolism by inhibiting the lipoxygenase and cyclooxygenase pathways (Huang 1992d). Coadministration of CUR with TPA in the priming phase significantly inhibited H_2O_2 production in mouse skin (Table IV-2). These results strongly suggested that anti-inflammatory effect of the curcuminoids, which regulate the infiltration of ROS-producing leukocytes, is a action mechanism for the inhibitory effects of TPA-induced H_2O_2 production in mouse skin. Further, coadministration of CUR with second TPA treatment also significantly inhibited H_2O_2 production to the same level as in the case of coadministration with first TPA treatment (Table IV-2). These findings appear to be consistent with a mechanism by which the curcuminoids inhibit H_2O_2 production, in part, via inhibition of leukocyte activation. Finally it should be noted that, needless to say, antioxidative activity of the curcuminoids against non-enzymatic formation of peroxides or reactive carbonyl compounds might, to a certain extent, participate in the antioxidative mechanism in mouse skin.

In summary, the author demonstrated the inhibitory effects of some chemopreventive agents on TPA-induced H_2O_2 production in mouse skin. Based on the double TPA application model, it was possible to classify the chemopreventive agents into 3 types of oxidative stress inhibitors; 1) priming phase-specific inhibitor, 2) activation phase-inhibitor, and 3) non-phase-specific inhibitor. It should be noted that the use of such profiles is quite effective to predict inhibitory activities in some *in vitro* assays such as arachidonate metabolism and O_2^- generation. Thus, the double TPA application model is clarified first in the present study to be very useful not only for the identification of effective chemopreventers but also for the investigation of their action mechanism.

Experimental

Chemicals

AUR and UMB were isolated from the cold-pressed oil of *natsumikan* (*Citrus natsudaidai* HAYATA) as previously reported (Murakami 1997). PPBa was kindly supplied by Toyo-Hakka Co. Ltd. (Okayama, Japan). CUR was purified by preparative silica gel TLC from commercial turmeric (Diawa Kasei Co., Saitama, Japan). THC and DHTHC were prepared by hydrogenation with PtO_2 of CUR or demethylation using BBr_3 as previously reported (Osawa 1995, Nakayama 1997). All other chemicals were obtained as described in Chapter II.

Inhibitory Test of TPA-induced O_2^- Generation in Differentiated HL-60 Cells

See Chapter II.

Treatment of Animals

See Chapter III.

Determination of H_2O_2 in Mouse Skin

See Chapter III.

Anti-inflammation Test in Mouse Skin

See Chapter III.

Chapter V:
Inhibitors of Superoxide Generation in Leukocytes from *Artemisia lactiflora*

Introduction

Tumor promoter-induced ROS generation has been considered to play important roles in tumor promotion (Chapter I). In particular, TPA-type tumor promoters are reported to trigger O_2^- generation in epithelial cells and leukocytes through the XA/XOD and NADPH oxidase systems, respectively. Yoon *et al.* (1993) advocated a close relationship between the generation of ROS including O_2^- by phagocytic cells in inflammatory processes and tumor promotion. In this context, the author recognized O_2^- generation inhibitor to be effective and promising candidate for preventer of oxidative stress-related disease including cancer, because O_2^- is one precursor of some types of ROS. The author has demonstrated that some chemopreventive phytochemicals inhibited O_2^- generation in leukocytes *in vivo*, suggesting the O_2^- generation blockage is one of the important action mechanisms for their anti-tumor promotion (Chapters III and IV).

Based upon this, exploration of food phytochemicals with O_2^- generation inhibition in leukocytes *in vitro* were conducted. In this chapter, a chemical study on the active constituents containing both diacetylene and spiroketal moieties from *Artemisia lactiflora* (Compositae), an edible plant in southeast Asia, is described. Next, their marked inhibitory activities against tumor promoter TPA-induced O_2^- generation in differentiated HL-60 cells are reported. The structure-activity relationships of this series of compounds on O_2^- generation inhibition are also discussed.

Results and Discussion

Isolation of O_2^- Generation Inhibitors

In the screening test of a variety of edible plants for O_2^- generation inhibition in differentiated HL-60 cells, the methanol (MeOH) extract of *A. lactiflora* showed the highest inhibitory activity. The active constituents were traced by the inhibition test of TPA-induced O_2^- generation in differentiated HL-60 cells. Fresh leaves of *A. lactiflora* (1 kg) were extracted with MeOH, and the extract (30 g) was partitioned between ethyl acetate

(EtOAc) and water. The active EtOAc layer was chromatographed on silica gel to give active fractions eluted with 2.5-5% MeOH in $CHCl_3$. The active combined fraction was further separated on silica gel (*n*-hexane/EtOAc, stepwise) and then on ODS gel (MeOH/H_2O, stepwise). The final purification was done by preparative HPLC on Nova Pak C_{18} (acetonitrile: H_2O = 7: 3) to afford six colorless compounds (**1**; 23 mg, **2**; 5 mg, **3**; 72 mg, **4**; 10 mg, **5**; 7 mg, **6**; 72 mg).

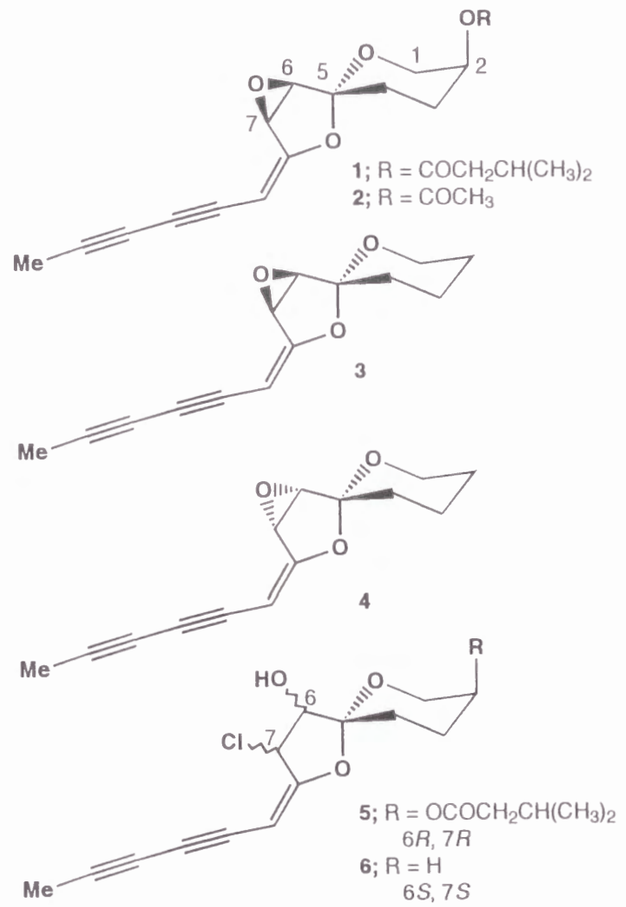

Fig. V-1 Structures of **1**-**6**

Structure-elucidation

Three (**1**, **2** and **3**) of the six compounds purified have already been isolated from this species by Bohlmann *et al.* (1982) and Birnecker *et al.* (1988). Also their structures including absolute stereochemistry, except for the configuration of the epoxide moiety in **1**, were confirmed as shown Fig. V-1. On the other hand, the three remaining compounds (**4**, **5** and **6**) were concluded to be new ones on the basis of their spectral data.

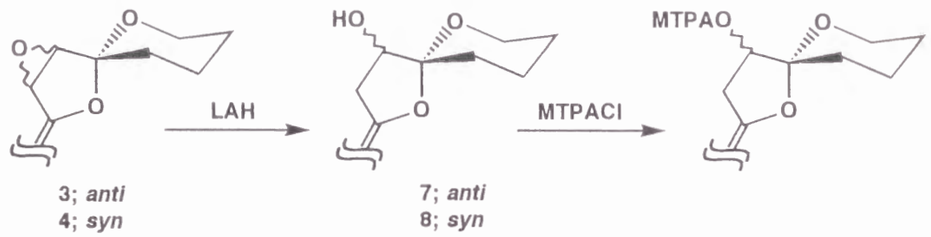

Fig. V-2 Synthesis of MTPA-esters of **7** and **8**

Table V-1 ^{1}H NMR data for MTPA esters of **7**[a] and its LIS data

	Advanced Mosher's method			LIS data
Position	(*S*)-MTPA-ester, δ	(*R*)-MTPA-ester, δ	Δδ δ*S*-δ*R*	Δδ
1 *ax*	3.71-3.78	3.72-3.79	-0.01	0.10
1 *eq*	-	-	-	0.14
4	-	-	-	0.43
6	5.16	5.17	-0.01	0.61
7 α	3.30	3.28	0.02	0.43
7 β	3.24	3.22	0.02	0.23
9	5.01	4.94	0.07	0.15
14	1.98	1.98	0	-

[a]In ppm from internal TMS in $CDCl_3$.

Table V-2 ^{1}H NMR data for MTPA esters of **8**[a]

Position	(*S*)-MTPA-ester, δ	(*R*)-MTPA-ester, δ	Δδ δ*S*-δ*R*
1 *ax*	3.80-3.84	3.72-3.79	0.04
6	5.20	5.11	0.09
7 α	3.26	3.36	-0.10
7 β	3.20	3.30	-0.10
9	4.96	4.96	0
14	1.97	1.97	0

[a]In ppm from internal TMS in $CDCl_3$.

Compound **4** ($C_{14}H_{14}O_3$, $[\alpha]_D^{21}$ −276.0°, *c* 0.10, $CHCl_3$) was suggested to be a diastereomer of **3** ($C_{14}H_{14}O_3$, $[\alpha]_D^{28}$ −26.5°, *c* 0.50, $CHCl_3$), on the basis of the MS, ^{1}H NMR and optical rotation data. The absolute configurations at C-6 of **3** and **4** were examined by the advanced Mosher's method (Fontana 1996, Sullivan 1973). Lithium aluminum hydride (LAH) reductions of **3** and **4** gave the corresponding 6-carbinols (**7** and **8**, respectively) which were then converted to (*R*)- and (*S*)-α-methoxy-α-trifluoromethylphenylacetate (MTPA) esters. Chemical shift differences (Δδ; δ*S*-δ*R*) between *S*- and *R*-esters of **7** showed positive values on H-7 and H-9, while a negative value was shown for H-1 (Table V-1). On the other hand, the data from the esters of **4** were reversed (H-7; negative, H-1; positive) as shown in Table V-2. These ^{1}H NMR analyses clearly indicated that the absolute configurations at C-6 of **3** and **4** are *R* and *S*, respectively. The ^{1}H NMR data of **7** using lanthanide-induced shift (LIS) reagent allowed the author to determine the relative configuration concerning *anti* or *syn* between the hydroxyl group at C-6 and the oxygen of tetrahydropyran. As for **7**, the signals at H-6, H-4 *equatorial*, and H-7α were significantly shifted downfield (Δδ 0.43-0.61 ppm). The LIS value for H-4 *equatorial* (Δδ 0.43 ppm) was larger than those of H_2-1 (Δδ 0.14, 0.10 ppm) (Table V-1). This result suggested that the oxygen of the tetrahydropyran is oriented in the *anti*-direction to the hydroxyl at C-6. On the basis of these data, the absolute configurations at C-5, 6, and 7 of **3** were determined to be *R*, *R*, and *S*, respectively.

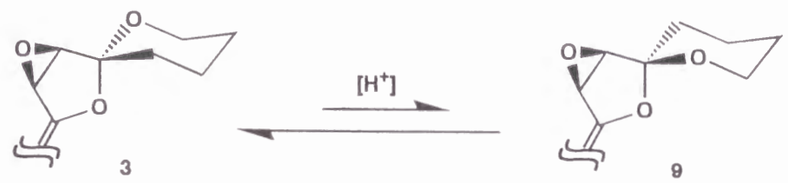

Fig. V-3 Isomerization of **3** to **9** in the acidic condition

Acid treatment of **3** gave **9** (Fig. V-3), an isomerized product at C-5 (Birnecker 1988), whose optical rotation (+250.7°) showed an opposite sign to that of **4** (−259.8°), indicating evidently that **4** is the diastereomeric isomer of **3** only in the epoxide orientation.

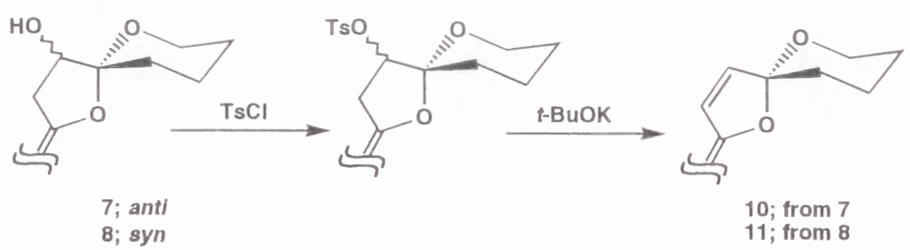

Fig. V-4 Synthesis of **10** and **11**

This was further supported by a double bond formation reaction of **7** and **8**, as shown in Fig. V-4. The tosylates of 6-carbinols, **7** and **8**, were treated with base to give the corresponding diene spiroketals **10** and **11**, respectively. The retention time on HPLC and ^{1}H NMR data of **11** from **8** were in good agreement with those of **10** from **7**. Interestingly, easy stereo-isomerization of the exo double bond at C-8 under light was detected (Fig. V-5). Therefore, it was necessary to treat carefully the diene spiroketal in the dark. Thus the stereochemistry of **4** (5*R*, 6*S*, and 7*R*) was first established.

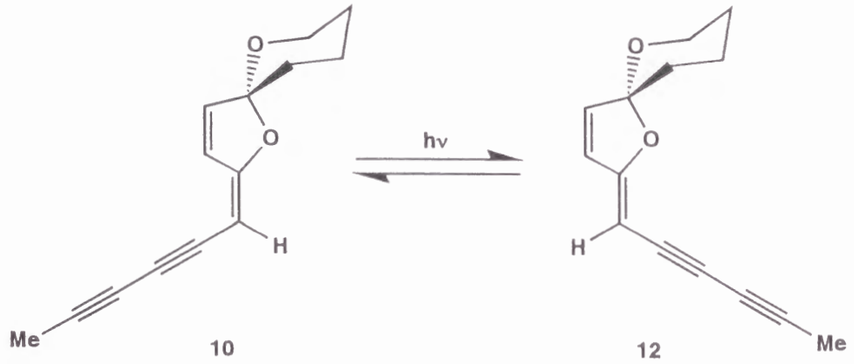

Fig. V-5 Stereo-isomerization of **10** to **12**

The NMR data of **5** and **6** were similar to those of **1** and **3** (or **4**), respectively, except for the chemical shifts of H-6 and H-7. The chemical shifts of both H-6 and H-7 of **5** (δ 4.09 and 4.78, respectively) and **6** (δ 4.03 and 4.76, respectively) were significantly shifted downfield by 0.2-0.4 ppm as compared with those of **1** and **3** (or **4**). The acetylation of **6** afforded a monoacetate whose ^{1}H NMR showed a downfield shift of C-6 (δ 5.26), suggestive of the existence of a hydroxyl group at C-6 in **6**. The molecular

formulae suggested **5** and **6** ($C_{19}H_{23}O_5Cl$ and $C_{14}H_{15}O_3Cl$, respectively) to be chlorohydrine forms of **1** and **3** (or **4**), respectively.

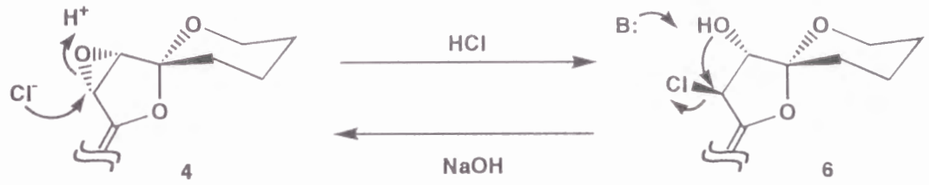

Fig. V-6 HCl treatment of **4** and NaOH treatment of **6**

Table V-3 ^{1}H NMR LIS data for **5**[a]

Position	**5**, δ^{1}H	**5** + Eu(fod)$_3$, δ^{1}H	Δδ^{1}H
1 *ax*	4.03	4.21	0.18
1 *eq*	3.94	4.16	0.22
2	4.90	5.40	0.50
4 *eq*	2.11	2.41	0.30
6	4.09	4.43	0.34
7	4.78	5.04	0.26
9	5.21	5.26	0.05

[a]In ppm from internal TMS in $CDCl_3$.

Alkaline treatment of **5** and **6** afforded to **1** and **4**, respectively, and inversely, treatment of **1** and **4** with hydrochloric acid again gave the corresponding chlorohydrine **5** and **6** as main products, respectively (Fig. V-6). Since chloride ion attacks the carbon to the opposite side of the epoxide at C-7, the absolute configurations of **6** at C-5, 6, and 7 were revealed as *R*, *S*, and *S*. On the other hand, although the absolute configuration at C-5 in **1** has already been confirmed to be *R* by X ray analysis and circular dichroism measurement, the absolute configurations of the epoxide forming carbons in **1** have not been confirmed yet (Bohlmann 1982). Next the ^{1}H NMR LIS data of **5** allowed the author to determine the relative configuration concerning *anti* or *syn* between the hydroxyl group at C-6 and the oxygen of tetrahydropyran in the same manner as in the case of **7** as described above. The signals at H-6, H-4 *equatorial*, and H-7 were significantly shifted downfield (Δδ 0.26-0.34 ppm). The LIS value for H-4 *equatorial* (Δδ 0.30 ppm) was larger than those of H_2-1 (Δδ 0.18, 0.22 ppm)

(Table V-3). This result suggested that the oxygen of the tetrahydropyran is oriented in the *anti*-direction to the hydroxyl at C-6 of **5**. Thus, the absolute configurations of **5** and **6** at C-5, 6, and 7 were confirmed as *R*, *R*, and *R* and *R*, *S*, and *S*, respectively. On the basis of the stereochemistry of **5**, it is thus evident that the absolute configurations of **1** at C-6 and 7 are the same as those of **3** as shown in Fig. V-1 (*R*, *R*, and *S*, respectively).

There is some doubt that these chlorohydrines are naturally occurring although the related chlorohydrines are reported to be present in other families of Compositae plants, e.g., *Carthamus*, *Eclipta*, *Echinops*, etc., but not in *Artemisia* genera (Bohlmann 1973). However, it is important to note that they were detectable in the methanol extract of the fresh leaves by HPLC analysis (data not shown).

Inhibitory Activities against TPA-induced O_2^- Generation

Table V-4 Inhibitory effects of the acetylenes on TPA-induced O_2^- generation[a]

	% Inhibition at concentration of (μM)					
Compound	5	10	20	50	100	IC_{50} (μM)
1	28	60	81	>99	N.T.	7.6
2	N.T.	32	61	80	N.T.	29
3	13	15	23	51	77	47
4	N.T.	N.T.	23	55	78	43
5	N.T.	29	N.T.	67	75	28
6	N.T.	19	N.T.	47	60	56
7	N.T.	5	N.T.	14	24	>100
9	N.T.	19	N.T.	50	61	50
10	N.T.	6	N.T.	27	43	>100
12	N.T.	7	N.T.	28	40	>100
Genistein	N.T.	0	N.T.	30	49	102

[a]The maximal SD for each experiment was 5% from at least duplicate tests.

The inhibitory effects of the natural (**1**-**6**) and the related synthesized compounds (**7**, **9**, **10** and **12**) together with genistein on TPA-induced O_2^- generation are summarized in Table V-4. Compound **1** (IC_{50} = 7.6 μM) was determined to be a quite potent O_2^- generation inhibitor, whose activity was much stronger than that of genistein (IC_{50} = 102 μM). On the other hand, **3**, **4**, and **9** (IC_{50} = 47 μM, 43 μM, and 50 μM, respectively) showed 5 times weaker inhibitory activity than **1**. These

results suggested that an acyloxyl group at the C-2 position may be an enhancing factor for inhibition, while the absolute configurations are not important. The inhibitory activities of **10** (IC_{50} > 100 μM) and **12** (IC_{50} > 100 μM) were significantly lower than those of the corresponding epoxide **3**. Moreover, 6-carbinol **7** (IC_{50} > 100 μM) also showed much less inhibitory activity than **3**. These results clearly showed that the reductive ring opening of the epoxy group reduces inhibitory activity. In addition, the stereochemistry of exo double bond at C-8 is not important for the activity. The evaluation of the presence of the triple bonds for activity remains to be elucidated. In any case, while polyacetylenes are known to possess several biological roles in various ecosystems (nematicidal, antibiotic, insect repellent, etc.) (Yano 1983), the author first described inhibitory effects of the diacetylenes against TPA-induced O_2^- generation in differentiated HL-60 cells.

Experimental

General Procedure

Analytical instruments used were as follows: HPLC, HITACHI 655A-11; $[\alpha]_D$, Jasco Model J-5; UV, Shimadzu UV 200 and UV 2200AI; IR, Shimadzu IR-435; MS, JEOL JMS-DX 300, ^{1}H NMR; Bruker ARX500 and AC300 (ref. TMS).

*Isolation of Compound **1**-**6** from* Artemisia lactiflora

Fresh leaves of *A. lactiflora* (1 kg) were extracted with MeOH at room temperature, and the filtrate was concentrated *in vacuo* to give a green oily syrup. Further fractionation was carried out by monitoring the inhibitory activity of TPA-induced O_2^- generation in differentiated HL-60 cells. The extract (30 g) was partitioned between EtOAc and water. The active EtOAc layer was chromatographed on silica gel (Wako gel C-100) to give active 2.5-5% MeOH in $CHCl_3$ eluates. This fraction was further separated on silica gel (Wako gel C-200, Wako Pure Chemical Industry, *n*-hexane/EtOAc, stepwise) and then on ODS gel (YMC I-40/64 gel, Yamamura Chemical Lab., MeOH/H_2O, stepwise). The final purification was done by preparative HPLC on Nova Pak C_{18} (Waters, acetonitrile: H_2O = 7: 3) to afford six colorless compounds (**1**; 23 mg, **2**; 72 mg, **3**; 10 mg, **4**; 7

mg, **5**; 72 mg, **6**; 5 mg). Compound **1**, $[\alpha]_D^{31}$ −16.0° (*c* 0.41, $CHCl_3$). UV λmax (EtOH) nm (log ε): 217 (3.97), 225 (4.08), 266 (3.71), 279 (3.85), 294 (3.78). IR νmax (KBr) cm^{-1}: 2900, 2140, 1700, 1650, 1200. ^1H NMR (500 MHz, $CDCl_3$) δ: 0.98 (6H, *d*, *J* = 6.7 Hz, 4'-H), 1.98 (3H, *s*, 14-H), 2.10 (5H, *m*), 2.25 (2H, *d*, *J* = 7.0 Hz, 2'-H), 3.86 (1H, *d*, *J* = 2.8 Hz, 6-H), 3.95 (1H, *dd*, *J* = 14.0, 2.0 Hz, 1β-H), 4.02 (1H, *d*, *J* = 13.8 Hz, 1α-H), 4.31 (1H, *d*, *J* = 2.7 Hz, 7-H), 4.92 (1H, *brs*, 2-H), 5.16 (1H, *brs*, 9-H). EIMS (probe, 70eV) *m/z*: 330 ($[M]^+$, $C_{19}H_{22}O_5$). Compound **2**, $[\alpha]_D^{23}$ −35.1° (*c* 0.23, $CHCl_3$). UV λmax (EtOH) nm (log ε): 216 (4.40), 222 (4.51), 263 (4.14), 277 (4.29), 293 (4.21). IR νmax (KBr) cm^{-1}: 2920, 2140, 1730, 1650. ^1H NMR (500 MHz, $CDCl_3$) δ: 1.99 (3H, *d*, *J* = 1.1 Hz, 14-H), 2.13 (3H, *s*, 2'-H), 3.80 (1H, *ddd*, *J* = 1.7, 1.7, 11.2 Hz, 1β-H), 3.89 (1H, *d*, *J* = 2.7 Hz, 6-H), 3.99 (1H, *dd*, *J* = 12.4, 1.8 Hz, 1α-H), 4.31 (1H, *d*, *J* = 2.7 Hz, 7-H), 4.88 (1H, *brs*, 2-H), 5.19 (1H, *brs*, 9-H). EIMS (probe, 70eV) *m/z*: 288 ($[M]^+$, $C_{16}H_{16}O_5$). Compound **3**, $[\alpha]_D^{28}$ −26.5° (*c* 0.50, $CHCl_3$). UV λmax (EtOH) nm (log ε): 214 (4.36), 222 (4.48), 262 (4.10), 276 (4.25), 290 (4.17). IR νmax (KBr) cm^{-1}: 2900, 2140, 1650. ^1H NMR (500 MHz, $CDCl_3$) δ: 1.62-1.93 (6H, *m*), 1.98 (3H, *s*, 14-H), 3.77 (1H, *dd*, *J* = 10.0, 4.5 Hz, 1β-H), 3.80 (1H, *d*, *J* = 2.7 Hz, 6-H), 3.86 (1H, *ddd*, *J* = 12.0, 10.0, 3.0 Hz, 1α-H), 4.29 (1H, *d*, *J* = 2.7 Hz, 7-H), 5.18 (1H, *brs*, 9-H). EIMS (probe, 70eV) *m/z*: 230 ($[M]^+$, $C_{14}H_{14}O_3$). Compound **4**, $[\alpha]_D^{21}$ −259.8° (*c* 0.08, $CHCl_3$). UV λmax (EtOH) nm (log ε): 216 (4.17), 225 (4.15), 265 (3.94), 278 (4.11), 293 (4.01). IR νmax (KBr) cm^{-1}: 2900, 2140, 1640. ^1H NMR (500 MHz, $CDCl_3$) δ: 1.60-1.98 (6H, *m*), 1.98 (3H, *s*, 14-H), 3.79 (1H, *d*, *J* = 2.8 Hz, 6-H), 3.85-3.93 (2H, *m*, 1-H), 4.28 (1H, *d*, *J* = 2.8 Hz, 7-H), 5.15 (1H, *brs*, 9-H). EIMS (probe, 70eV) *m/z*: 230 ($[M]^+$, $C_{14}H_{14}O_3$). Compound **5**, $[\alpha]_D^{24}$ +290.2° (*c* 0.10, $CHCl_3$). UV λmax (EtOH) nm (log ε): 204 (4.15), 219 (4.27), 261 (4.04), 276 (4.21), 289 (4.17). IR νmax (KBr) cm^{-1}: 3400, 2950, 2140, 1720, 1640. ^1H NMR (500 MHz, $CDCl_3$) δ: 0.97 (3H, *d*, *J* = 6.7 Hz, 4'-H), 1.99 (3H, *s*, 14-H), 2.08-2.18 (5H, *m*), 2.24 (2H, *d*, *J* = 7.0 Hz, 2'-H), 2.87 (1H, *d*, *J* = 7.5 Hz, -OH), 3.94 (1H, *d*, *J* = 12.8 Hz, 1β-H), 4.03 (1H, *dd*, *J* = 12.8, 1.4 Hz, 1α-H), 4.09 (1H, *dd*, *J* = 7.3, 4.5 Hz, 6-H), 4.78 (1H, *dd*, *J* = 4.5, 1.4 Hz, 7-H), 4.90 (1H, *s*, 2-H), 5.21 (1H, *s*, 9-H). HR-EIMS (probe, 70eV) *m/z*: 366.1227 ($[M]^+$, calcd. for $C_{19}H_{23}O_5Cl$, 366.1234). Compound **6**, $[\alpha]_D^{24}$ +342.9° (*c* 0.70, $CHCl_3$). UV λmax (EtOH) nm (log ε): 205 (4.18), 225 (4.28), 265 (4.07), 280 (4.23), 294 (4.20). IR νmax (KBr) cm^{-1}: 3450, 2950, 2140, 1640. ^1H

NMR (500 MHz, $CDCl_3$) δ: 1.60-1.98 (6H, *m*), 1.98 (3H, *s*, 14-H), 2.87 (1H, *d*, *J* = 7.5 Hz, -OH), 3.85-3.93 (2H, *m*, 1-H), 4.03 (1H, *dd*, *J* = 6.9, 4.6 Hz, 6-H), 4.76 (1H, *dd*, *J* = 4.5, 1.4 Hz, 7-H), 5.19 (1H, *s*, 9-H). HR-EIMS (probe, 70eV) *m/z*: 266.0708 ($[M]^+$, calcd. for $C_{14}H_{15}O_3Cl$, 266.0710).

*LAH Reduction of **3** and **4***

Compound **3** (6.1 mg, 26.5 μmol) in dry diethyl ether (Et_2O) was treated with LAH (1 mg, 26.4 μmol). After 1 h, a small amount of water was added and the mixture was filtered and extracted with Et_2O. The organic layer was dried over Na_2SO_4 and concentrated. The residue was chromatographed on silica gel with *n*-hexane-EtOAc (4: 1) to give **7** (3.0 mg, 12.9 μmol, 49% yield). Compound **7**, ^1H NMR (500 MHz, $CDCl_3$) δ: 1.58-2.02 (6H, *m*), 1.97 (3H, *d*, 14-H), 2.77 (1H, *d*, *J* = 17.7 Hz, 7*cis*-H), 3.14 (1H, *ddd*, *J* = 17.7, 5.5, 2.6 Hz, 7*trans*-H), 3.68 (1H, *ddd*, *J* = 11.0, 2.5, 2.5 Hz, 1β-H), 3.78 (1H, *ddd*, *J* = 11.0, 11.0, 3.8 Hz, 1α-H), 4.02 (1H, *t*, *J* = 5.5 Hz, 6-H), 5.00 (1H, *brs*, 9-H). EIMS (probe, 70eV) *m/z*: 232 ($[M]^+$, $C_{14}H_{16}O_3$).

A solution of **4** (8.9 mg, 38.7 μmol) in dry Et_2O was treated with LAH (1.5 mg, 39.6 μmol). After 40 min, a small amount of water was added and the mixture was filtered and extracted with Et_2O. The organic layer was dried over Na_2SO_4, concentrated and chromatographed on silica gel to give **8** (5.2 mg, 22.4 μmol, 58% yield). Compound **8**, ^1H NMR (500 MHz, $CDCl_3$) δ: 1.60-1.68 (6H, *m*), 1.97 (3H, *d*, *J* = 1.0 Hz, 14-H), 2.67 (1H, *ddd*, *J* = 15.2, 8.8, 2.5 Hz, 7*cis*-H), 3.16 (1H, *ddd*, *J* = 16.9, 7.7, 1.4 Hz, 7*trans*-H), 3.82-3.86 (2H, *m*, 1-H), 3.90 (1H, *brd*, *J* = 7.0 Hz, 6-H), 4.91 (1H, *brs*, 9-H). EIMS (probe, 70eV) *m/z*: 232 ($[M]^+$, $C_{14}H_{16}O_3$).

Preparation of MTPA Esters

Compound **7** (2.6 μg, 11.2 mmol) was dissolved in dry pyridine (50 μl) and (*R*)-(-)-MTPA chloride (5 μl) was added. After 30 min, *N*,*N*-dimethyl-1,3-propanediamine (5 μl) was added and then incubated another 10 min. The mixture was extracted with EtOAc. The organic layer was dried over Na_2SO_4 and concentrated. The product was chromatographed on silica gel with *n*-hexane-EtOAc (9: 1) to give the (*S*)-(-)-MTPA ester of **7** (3.8 mg, 8.0 μmol, 71% yield). In the same way, the (*R*)-(+)-MTPA ester of **7** (5.2 mg, 11.9 μmol, 92% yield) from **7** (3.0 mg, 12.9 μmol), the (*S*)-(+)-

MTPA ester of **8** (2.5 mg, 5.3 μmol, 88% yield) from **8** (1.4 mg, 6.0 μmol), and the (*R*)-(+)-MTPA ester of **8** (2.6 mg, 6.0 μmol, 92% yield) from **8** (1.5 mg, 6.5 μmol) were prepared, respectively.

NMR Experiments

For the determination of the LIS values, increasing amounts of $Eu(fod)_3$ or $Eu(dpm)_3$ was added to a solution of **5** or **7** (3 mg in 0.5 ml $CDCl_3$). The LIS for a concentration ratio of **5**: $Eu(fod)_3$ or **7**: $Eu(dpm)_3$ = 1: 1 were obtained by extrapolation of 4 different reagent conditions.

*Isomerization of **3** to **9***

Isomerization of **3** was done by the method previously reported (Birnecker 1988). Compound **3** (4.2 mg, 17.8 μmol) was dissolved in MeOH/Et_2O = 3: 1, to which *p*-toluenesulfonic acid (1 mg, 5.26 μmol) was added. After 6 h, the products were purified by preparative TLC (*n*-hexane/EtOAc =4: 1) to give **9** (0.6 mg, 2.60 μmol, 14.6% yield). $[\alpha]_D^{23}$ +250.7° (*c* 0.08, $CHCl_3$). ^1H NMR (300 MHz, $CDCl_3$) δ: 1.61-1.94 (6H, *m*), 1.98 (3H, *s*, 14-H), 3.79 (1H, *d*, *J* = 2.8 Hz, 6-H), 3.86-3.91 (2H, *m*, 1-H), 4.29 (1H, *d*, *J* = 2.8 Hz, 7-H), 5.15 (1H, *brs*, 9-H).

*Reduction of Epoxide in **3** and **4***

Compound **7** (2.6 mg, 11.2 μmol) and *p*-toluenesulfonyl chloride (20 mg, 105.3 μmol) in pyridine were stirred at room temperature for 3 h. The solution was poured into ice-cold water and extracted with EtOAc. The organic layer was washed with water and dried over Na_2SO_4. The reaction mixture was purified by preparative TLC (*n*-hexane/EtOAc =4: 1) to give *p*-toluenesulfonyl ester of **7** (1.9 mg, 2.60 μmol). To a DMSO solution of the *p*-toluenesulfonyl ester was added potassium *t*-butoxide (1.5 mg, 13.4 μmol), and the mixture was stirred at room temperature for 24 h. The reaction mixture was separated by preparative TLC (*n*-hexane/EtOAc = 9: 1) to give **10** (0.4 mg, 1.89 μmol, 17% yield) and **12** (0.4 mg, 1.89 μmol, 17 % yield). Compound **10**, ^1H NMR (500 MHz, $CDCl_3$) δ: 1.42-1.89 (6H, *m*), 1.98 (3H, *d*, *J* = 1.0 Hz, 14-H), 3.82-4.02 (2H, *m*, 7-H), 4.97 (1H, *brs*, 9-H), 6.22 (1H, *dd*, *J* = 5.9, 1.7 Hz, 6-H), 6.65 (1H, *d*, *J* = 5.9 Hz, 7-H). EIMS (probe, 70eV) *m/z*: 214 ($[M]^+$, $C_{14}H_{14}O_3$). Compound **12**, ^1H NMR (500 MHz, $CDCl_3$) δ: 1.42-1.89 (6H, *m*), 1.98 (3H, *d*, *J* = 1.0 Hz, 14-H),

3.82-4.02 (2H, *m*, 1-H), 4.61 (1H, *brs*, 9-H), 6.17 (1H, *d*, *J* = 5.6 Hz, 6-H), 6.19 (1H, *d*, *J* = 5.6 Hz, 7-H). EIMS (probe, 70eV) *m/z*: 214 ($[M]^+$, $C_{14}H_{14}O_3$).

In the same manner, compound **8** (3.0 mg, 12.9 μmol) was treated with *p*-toluenesulfonyl chloride and potassium *t*-butoxide to give **11** (0.6 mg, 2.84 μmol, 22% yield) and **12** (0.5 mg, 2.37 μmol, 18% yield). Analytical HPLC of both **10** and **11** on YMC ODS(A) (mobile phase: 60% CH_3CN in H_2O, flow rate: 1.0 ml/min, detected by UV 254 nm) showed a peak at t_R 12.5 min.

*Acetylation of **6***

Compound **6** (4.72 mg, 17.7 μmol) in dry pyridine (0.5 ml) and acetic anhydride (0.05 ml) was allowed to stand at room temperature for 4 h. The solution was poured onto ice-cold water and extracted with EtOAc. The organic layer was washed with water, dried over Na_2SO_4 and purified by preparative HPLC on μBondasphere C_{18} (75% acetonitrile in water) to give an acetate of **6** (3.8 mg, 12.3 μmol, 69% yield). IR νmax (KBr) cm^{-1}: 2950, 2140, 1735, 1640. ^1H NMR (300 MHz, $CDCl_3$) δ: 1.56-1.87 (6H, *m*), 1.99 (3H, *d*, *J* = 1.2 Hz, 14-H), 2.17 (3H, *s*, 2'-H), 3.80-3.85 (2H, *m*, 1-H), 4.97 (1H, *dd*, *J* = 3.5, 1.7 Hz, 7-H), 5.22 (1H, *s*, 9-H), 5.26 (1H, *d*, *J* = 5.2 Hz, 6-H).

*Alkaline Treatment of **5** and **6***

To a MeOH (1 ml) solution of **5** (2.6 mg, 7.1 μmol) was added 0.01% NaOH (0.2 ml). The mixture was stirred at 40°C for 4 h, then diluted with water and extracted with EtOAc. The organic layer was washed with water, dried over Na_2SO_4 and purified by preparative TLC (*n*-hexane/EtOAc = 4: 1) to give **1** (1.2 mg, 3.6 μmol, 51% yield).

Compound **6** (2.5 mg, 9.4 μmol) was treated in the same manner to give **4** (1.0 mg, 4.3 μmol, 46% yield).

*Acidic Treatment of **1** and **4***

To a MeOH (0.2 ml) solution of **1** (0.5 mg, 1.5 μmol) or **4** (0.5 mg, 2.2 μmol) was added 10% HCl (80 μl). The mixture was stirred at room temperature for 10 min, then diluted with water and extracted with EtOAc. The organic layer was washed with a saturated $NaHCO_3$ solution, dried

over Na_2SO_4 and concentrated. Analytical HPLC of the products from **1** on YMC ODS(A) (mobile phase: 65% CH_3CN in H_2O, flow rate: 1.0 ml/min, detected by UV 254 nm) showed a main peak at t_R 12.5 min, which are in good agreement with that of **5**. In the same way, analytical HPLC of the products from **4** on YMC ODS(A) (mobile phase: 60% CH_3CN in H_2O, flow rate: 1.0 ml/min, detected by UV 254 nm) showed a main peak at t_R 9.5 min, which are in good agreement with that of **6**.

Inhibitory Test of TPA-induced O_2^- Generation in Differentiated HL-60 cells

See Chapter II.

Chapter VI:
Inhibitory Effects of AL-1 on Reactive Oxygen Species Generation and Tumor Promotion in ICR Mouse Skin

Introduction

In Chapter V, the author demonstrated the isolation and identification of new O_2^- generation inhibitors in leukocytes from *A. lactiflora*. To prove the hypothesis that ROS generation inhibitors suppress oxidative stress and thus tumorigenesis in rodents effectively (Chapter I), the inhibitory effects of compound **1** (AL-1) on TPA-induced H_2O_2 production and tumor promotion in mouse skin were investigated.

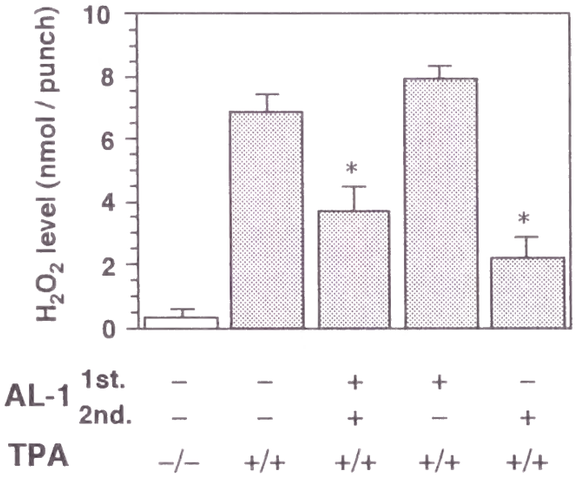

Fig. VI-1 Inhibitory effects of AL-1 on double TPA application-induced H_2O_2 production in mouse skin

ICR mice were treated by a double treatment protocol as described here and in "**Experimental**". Mouse skins were treated with AL-1 (810 nmol) or acetone 30 min before 1st and/or 2nd TPA treatment. The mice were sacrificed 1 h after the second TPA application, and their skins were removed for H_2O_2 assays. Statistical significance was determined by the Student's *t*-test and is expressed as *, Inhibitor/TPA *versus* TPA.

Results

Inhibitory Effects of AL-1 on TPA-induced H_2O_2 Production in Mouse Skin

As shown in Fig. VI-1, double applications of 8.1 nmol TPA at a 24-h interval increased the level of H_2O_2 by about 20-fold (6.84 ± 0.60 nmol / skin punch) to that in the control mice which were treated twice only with acetone instead of TPA. When AL-1 (810 nmol) almost significantly

inhibited H_2O_2 production (3.69 ± 0.76 nmol / skin punch, IE = 46%). To distinguish whether AL-1 inhibit the priming or activation phase in a double TPA application model, experiments were conducted in which AL-1 was coadministered with either the first (priming) or second (activation) dose of TPA. Fig. VI-1 shows the inhibitory effect of AL-1 applied prior to either first or second TPA treatment on H_2O_2 generation in skin. A dramatic decrease in the H_2O_2 level was observed in the mice to which ACA was coadministered in the activation phase (2.27 ± 0.59 nmol / skin punch, inhibitory effect; 67%). On the contrary, AL-1 applied in the priming phase exhibited no inhibition of H_2O_2 generation, similar to that in ACA and AUR.

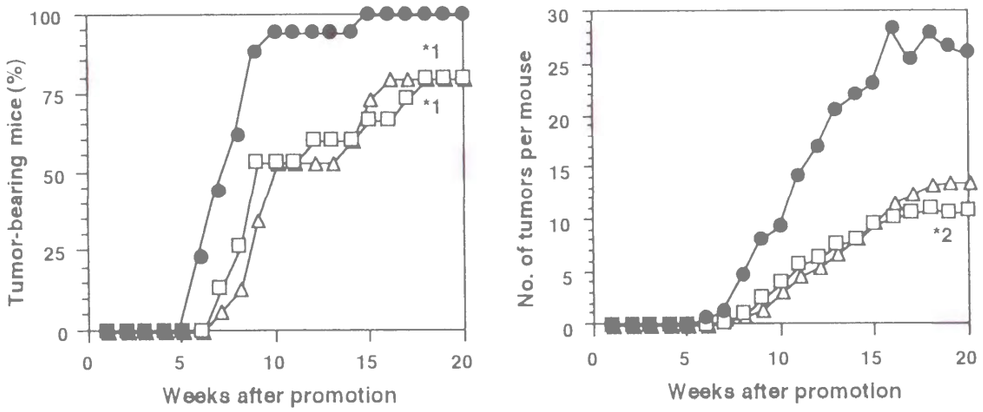

Fig. VI-2 Anti-tumor promoting activity of AL-1

One group was composed of 15 female ICR mice. The mice at 7 weeks old were initiated with DMBA (0.19 μmol). One week after initiation, the mice in group 1 (filled circles) were promoted with TPA (1.6 nmol/0.1 ml in acetone) twice a week for 20 weeks. In the AL-1-treated experiments, the mice in group 2 (open squares) and 3 (open triangles) were treated with AL-1 (16 nmol and 160 nmol/0.1 ml in acetone, respectively) 40 min prior to each TPA treatment. The anti-tumor promoting activity was evaluated by both the ratio of tumor-bearing mice (left) and the number of tumors per mouse (right). Statistical analysis was done by the χ^2-test on tumor-bearing mouse and the Student's *t*-test on the number of tumors per mouse. $^{*1}P < 0.005$, $^{*2}P < 0.01$.

Anti-tumor Promoting Activity of AL-1 in ICR Mouse Skin

Anti-tumor promoting activity of AL-1 was then examined by a two-stage carcinogenesis experiment in mouse skin. As shown in Fig. VI-2, tumors began to appear at 6 weeks after tumor promotion by TPA. The ratio of tumor-bearing mice and the average number of tumors per mouse in the control group reached 100% and 25.8, respectively, at the final week (20 weeks) of the experiment. In the group treated with 160 nmol of AL-1 40 min prior to each TPA treatment, both the average number of tumors per mouse and the ratio of tumor-bearing mice were reduced by 58% ($P<0.01$ in *t*-test) and 20% ($P < 0.005$ in χ^2-test), respectively. AL-1 even at 16 nmol significantly reduced the ratio of tumor-bearing mice by 20% ($P< 0.005$ in χ^2-test).

Discussion

In this chapter, the author demonstrates the potent inhibitory effects of the O_2^- generation inhibitor AL-1 on H_2O_2 production in mouse skin. No inhibitory effect of AL-1 on the TPA-induced priming phase was confirmed and strong inhibition of the activation phase was clearly observed (Fig. VI-1). In addition, AL-1 failed to inhibit single TPA application-induced edema formation in mouse ears (data not shown). These results conclude that AL-1 is an ACA-type ROS generation inhibitor which specifically inhibits the activation, but not infiltration, of leukocytes. Further AL-1 at 160 nmol significantly inhibited the average number of tumors per mouse in the two-stage mouse skin tumor promoting experiment (Fig. VI-2). This activity was comparable to those of chemopreventers from edible plants shown in Table IV-1. These results indicate that O_2^- generation inhibitors can be evaluated as new types of chemopreventers distinct from radical scavenger-type antioxidants. Thus regulation of leukocytes in inflammatory regions is proved to be one of the effective strategies for oxidative stress control and hence cancer chemoprevention.

Experimental

Chemicals and Animals

See Chapter III.

Treatment of Animals

See Chapter III.

Determination of H_2O_2 in Mouse Skin

See Chapter III.

Two-stage Carcinogenesis Experiment in ICR Mouse Skin

The anti-tumor promoting activity of AL-1 was examined by a standard initiation-promotion protocol with DMBA and TPA as previously reported (Murakami 1997). One group was composed of 15 female ICR mice housed 5 per cage. The mice were given commercial rodent pellets and fresh tap water *ad libitum*, both of which are freshly exchanged twice a week. The back of each mouse was shaved with a surgical clipper two days before initiation. The mice at 7 weeks old were initiated with DMBA (0.19 μmol/0.1 ml in acetone). One week after initiation, the mice were promoted with TPA (1.6 nmol/0.1 ml in acetone) twice a week for 20 weeks. In two other groups, the mice were treated with AL-1 (16 nmol or 160 nmol/0.1 ml in acetone) 40 min before each TPA treatment. The anti-tumor promoting activity was evaluated by both the ratio of tumor-bearing mice and the number of tumors, more than 1 mm in diameter, per mouse. The data were statistically analyzed using the Student's *t*-test for the average number of tumors per mouse and by the χ^2-test for the ratio of tumor-bearing mice.

Summary and Conclusion

Radical scavengers have been mainly noticed as candidates for chemopreventer because they strongly inhibit oxidative stress *in vitro* and *in vivo*. Therefore dietary radical scavengers, such as α-tocopherol, ascorbic acid, β-carotene and simple phenolics have so far attracted wide attention in recent years. β-Carotene is one of the most extensively studied agent for chemoprevention on account of its cancer preventive potencies in various animal models as well as epidemiological surveys. A puzzling outcome, however, was that β-carotene failed to reduce cancer risk and mortality in a recent clinical study. Moreover, some of the radical scavengers exert not only weak anti-tumor promoting activity but also carcinogenic activity in rodents when given at high doses. Hence, there may be a need to discover new types of chemopreventive agents which have antioxidative properties rather than radical scavenging activity by scrutinizing a diverse array of edible plants and their components.

On the basis of such context, the author proposes the hypothesis that ROS generation inhibitors, including enzyme induction inhibitors and/or enzyme activity inhibitors, suppress oxidative stress and thus tumorigenesis in rodents, more effectively than radical scavenging-type antioxidants because they can inhibit generation of some types of ROS at earlier stages and may not allow subsequent oxidative damage.

In Chapter II, the inhibitory effects of ACA on ROS generation in some biological systems *in vitro*, which is a potent chemopreventer of some organ carcinogeneses, were examined. The good structure-activity relationships of ACA analogs between EBV activation inhibition, O_2^- generation and intracellular peroxide formation in HL-60 cells were observed. Although ACA showed no radical scavenging potential, it strongly suppressed intracellular peroxide formation in HL-60 cells downstream of O_2^- generation, suggestive of the antioxidative potential of ACA in mouse skin. These results pointed out that inhibition of O_2^- generation in leukocytes, at least in part, might be an important action mechanism for suppression of oxidative stress and tumor promotion by ACA in mouse skin.

In Chapter III, to prove the above assumption directly, the author addressed whether or not ACA suppressed TPA-induced oxidative stress in mouse skin using a double TPA application model. The results obtained

provide steady evidence for the suppression of tumor promoter-induced H_2O_2 production by ACA in mouse skin. It is noteworthy that ACA exhibited no inhibitory effects on edema formation and the enhancement of MPO activity in the first TPA treatment. No inhibitory effect of ACA on the TPA-induced priming phase was clearly confirmed by an experiment in which ACA was coadministered only with the first dose of TPA (Fig. III-2). ACA showed no inhibitory effects on TPA-induced arachidonate release nor prostaglandin E_2 synthesis. Taken together, the inhibition of the priming event by ACA can be ruled out as the critical mechanism of H_2O_2 generation inhibition *in vivo*. On the other hand, coadministration of ACA only with the second TPA treatment successfully inhibited H_2O_2 production. There was a positive correlation between inhibition of the activation phase *in vivo* and suppression of leukocyte activation *in vitro*. The present data also indicate that ACA, a weak antioxidant in lipid peroxidation *in vitro* (in Chapter II), significantly inhibits TBARS formation in mouse epidermis, suggesting that suppression of leukocyte activation by ACA plays a critical role for its antioxidative effect in mouse skin. Further, the inhibitory effect of ACA on hyperplasia and mitosis of epidermal cells by double TPA applications, which give growth advantage to phenotypically altered populations, suggested that suppression of oxidative damage through ROS generation inhibition is an important action mechanism for its anti-tumor promotion properties.

Furthermore, histological studies clearly demonstrated that double TPA treatments enhance morphological changes of inflammatory responses. It should be noted that, although ACA can inhibit neither the arachidonate metabolism nor single application-induced edema formation, it can reduce the number of leukocytes in mouse cutis. To rationalize inhibition of leukocyte infiltration by ACA, it is important to note that O_2^- is known to participate in the formation of chemotactic factors and recruitment of PMNs. In any case, the author concluded that ACA potently suppresses TPA-induced oxidative stress and tumor promotion mainly via regulation of leukocytes including inhibition of ROS generation and interfering with their infiltration to the inflammatory regions of epidermis and dermis. Conversely, the present study strongly suggests that the double TPA application experiment is one of the most appropriate models not only to

predict the inhibitory potential of test compounds for chronic inflammation or tumor promotion but also to address action mechanisms.

In Chapter IV, an examination of the kind of events caused by TPA during the inflammation processes, priming and/or activation inhibitable by several phytochemicals was conducted to discuss the mechanism for oxidative stress control in mouse skin. The total inhibitory effects of most chemopreventers were significant (IE > 50%) and the specificities for inhibition in the priming or activation phase were quite different for each compound. Further mechanistic studies using *in vitro* assays of ROS generation and inflammation processes demonstrated quite clearly their action mechanisms through regulating inflammatory leukocytes for inhibition of double TPA treatment-induced oxidative damage in mouse skin.

Finally, exploration of new O_2^- generation inhibitors from edible plants was carried out (Chapter V). Three known diacetylenes and three novel related compounds with a chlorohydrin have been isolated from *Artemisia lactiflora*, an edible southeast Asian plant. Among the isolated constituents, the inhibitory effects of compound **1** (AL-1) on TPA-induced H_2O_2 production and tumor promotion in mouse skin were confirmed to support the hypothesis that ROS generating inhibitors suppress oxidative stress and thus tumorigenesis in rodents effectively. In Chapter VI, the author demonstrated that AL-1 is an ACA-type ROS generation inhibitor which especially inhibits activation, but not infiltration, of leukocytes. Further AL-1 at 160 nmol significantly inhibit mouse skin tumor promotion, to the extent comparable to that of typical chemopreventers from edible plants. These results indicated that O_2^- generation inhibitors may be evaluated as a new type of chemopreventer rather than as radical scavenger-type antioxidants.

In conclusion, the regulation of leukocytes in inflammatory regions is thus proved to be one effective strategy for oxidative stress control and cancer chemoprevention. This thesis proposed for the first time that suppression of the activation of inflammatory leukocytes generating O_2^- represents a new paradigm for cancer chemoprevention.

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List of Publications

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